Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Claims 3, 5, and 9 have been amended. Descriptive support for the amendments to claims 3 and 5 is provided in original claim 5 as well as page 7, lines 5–15, page 8, lines 1–8, and the Examples (*see* Example 8 re: adenocarcinoma). New claims 14–18 have been added. Descriptive support for new claims 14–16 is provided in claim 3. Descriptive support for new claims 17 and 18 is found on page 8, lines 27–32 of the specification as well as original claims 8 and 9. Therefore, no new matter has been introduced by these amendments.

Claims 3–9 and 12–18 are currently pending, with claims 3–5, 7–9, and 12–18 under examination, and claim 6 standing withdrawn. No excess claim fees are due with this submission.

This submission is accompanied by a petition for extension of time, a request for continue examination, and a Declaration of François Ichas, Ph.D. Under 37 C.F.R. § 1.132 ("Ichas Declaration"). All fees associated with this submission should be charged to deposit account 14-1138. Any overpayment or underpayment should be credited/charged to this same account.

The rejection of claims 3, 4, and 7 under 35 U.S.C. § 102(a) as anticipated by Murdaca et al., *AIDS* 16(2):304-5 (2002) ("Murdaca") is respectfully traversed.

At page 3 of the office action, the U.S. Patent and Trademark Office ("PTO") asserts that Kaposi's sarcoma ("KS") is an epithelial tumor. While applicants disagree with this interpretation by the PTO because Kaposi's sarcoma is a mesenchymal tumor, to clarify the claim language applicants have amended claim 3 to recite "carcinoma" rather than "epithelial tumor." KS is not a carcinoma, nor is it a tumor of the nervous system, a form of leukemia, or a fibro- or osteo-sarcoma. Therefore, the claims do not read on the treatment of KS as taught in Murdaca. For this reason, the rejection of claims 3, 4, and 7 as anticipated by Murdaca is improper and should be withdrawn.

The rejection of claims 8 and 9 under 35 U.S.C. § 103(a) for obviousness over Murdaca in view of U.S. Patent No. 6,235,733 to Bahal et al., ("Bahal") is respectfully traversed.

The teachings and deficiencies of Murdaca are noted above. As noted above, Murdaca discloses only the treatment of a patient suffering from AIDS and the related KS tumor.

There is no mention of any other types of tumor. Bahal is cited at page 10 of the office action for teaching oral liquid formulations of efavirenz; however, the PTO has failed to demonstrate how Bahal overcomes the above-noted deficiencies of Murdaca. Therefore, the obviousness rejection of claims 8 and 9, which ultimately depend from claim 3, is improper and should be withdrawn.

The rejection of claims 3, 4, and 7 under 35 U.S.C. § 103(a) for obviousness over Grimaudo *et al.*, *Eur. J. Cancer* 34:1756-1763 (1998) ("Grimaudo") is respectfully traversed for the reasons set forth in the accompanying Ichas Declaration.

Grimaudo investigated the cytotoxicity of 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole ("TBZ"), a non-nucleoside reverse transcriptase inhibitor ("NNRTI"), on human acute myeloid leukemic HL60 parental and drug resistant (HL60R) cell lines. Ichas Declaration at ¶ 5. Grimaudo showed that TBZ exhibits cytotoxic activity in the both HL60 and HL60R cells lines at a concentration of 50 μM (IC_{50}), a concentration that is 50-fold higher than the concentration of TBZ required to inhibit viral reverse transcriptase (RT) activity in several human primary cells and cell lines. *Id.* Since the cytotoxic effects of TBZ were observed at such a higher concentration than required to inhibit viral RT, it is highly unlikely that TBZ's cytotoxic activity in these cells is related to its viral RT inhibitory properties. *Id.*

That TBZ's mechanism of cytotoxicity is unrelated to its RT inhibitory activity is further supported by Grimaudo's observation that TBZ caused a selective induction of apoptosis in the HL60 drug resistant cell line compared to the parental cell line. Ichas Declaration at ¶ 6. While Grimaudo does not delineate TBZ's mechanism of cytotoxicity in the HL60 cell lines, based on TBZ's selective induction of apoptosis in the HL60R line, Grimaudo speculates a mode of action that involves bypassing drug resistance-related mechanisms such as the multidrug transporter or a differential interaction with topoisomerase II. *Id.*

The authors of Grimaudo, in consideration of the unique results obtained with TBZ, suggest that future studies should explore the mechanism of its anti-tumor and pro-apoptotic action. Ichas Declaration at ¶ 7. Grimaudo further suggests using TBZ derivatives that are metabolized more slowly than TBZ, and therefore retain their activity. *Id.* There is no suggestion in Grimaudo that other NNRTIs would have the same anti-tumor effect in the HL60 cells or other cancer cells. *Id.* Indeed, given the expectation that TBZ's effects were independent of its RT-inhibitory activity, Grimaudo's focus exclusively on TBZ derivatives

rather than on other NNRTI's is entirely reasonable. *Id.* Therefore, based on the data and conclusions of Grimaudo, one of skill in the art would have no reasonable expectation that NNRTIs, that are structurally unrelated to TBZ, such as nevirapine, efavirenz, delavirdine, and compounds in the class of 5,11-dihydro-6h-dipyrido[3,2-b:2',3'-e][1,4]diazepines, would exert similar cytotoxic effects in parental or drug-resistant cancer cell lines, simply based on their shared ability to inhibit viral RT. *Id.* None of the data presented by Grimaudo substantiates such a conclusion. *Id.*

In a subsequent publication by Grimaudo et al., "Apoptotic Effects of Thiazolobenzimidazole Derivatives on Sensitive and Multidrug Resistant Leukaemic Cells," *Eur. J. Cancer* 37(1):122–30 (2001) (Grimaudo 2001), the results from the continued investigation of the cytotoxic activity of TBZ derivatives on the same cell lines (as described in Grimaudo) are presented. Ichas Declaration at ¶ 8. Grimaudo 2001 reports that these compounds exert their anti-tumor effects by activating the programmed cell death pathway. *Id.* Grimaudo 2001 concludes that this activity is, at least in part, caspase mediated, as shown by the ability of caspase inhibitors to reduce the percentage of apoptosis induced by one of the TBZ derivatives. *Id.* These findings, which were published well in advance of the filing date of the above referenced patent application, further support that one of skill in the art would have no reasonable expectation, based on the teachings of Grimaudo, that NNRTIs that are structurally unrelated to TBZ, such as nevirapine, efavirenz, delavirdine, and compounds in the class of 5,11-dihydro-6h-dipyrido[3,2-b:2',3'-e][1,4]diazepines, would exhibit anti-tumor effects similar to that of TBZ. *Id.*

Moreover, one of skill in the art would not generalize the findings of Grimaudo, based on data obtained in one acute myeloid leukemic cell line, to solid tumors, such as carcinomas, tumours of the nervous system, and fibro- and osteo-sarcomas because of the fundamental differences in terms of the molecular mechanisms involved in the tumorigenic development of the two major categories of cancers. Ichas Declaration at ¶ 9. Furthermore, an illustration is the fact that strategies used for the therapeutic treatments of these two types of cancers are not similar. *Id.* However, this is not to be construed as any admission by applicants that the presently claimed invention is not enabling for treatment of leukemia. Indeed, the examples demonstrate as much using four different leukemia cell lines (*see* page 7, lines 5-15).

For the reasons discussed above, applicants disagree with the PTO's position that one of skill in the art would have found it obvious, based on the teachings of Grimaudo, to counteract the loss of cellular differentiation and to treat cell proliferation in tumor pathologies by administering to a subject a compound in the class of 5,11-dihydro-6h-dipyrido[3,2-b:2',3'-e][1,4]diazepines, nevirapine, efavirenz, delavirdine, or corresponding salts thereof as taught by and claimed in the above referenced patent application. Accordingly, the rejection of 3, 4, and 7 is improper and should be withdrawn.

The rejection of claims 3, 4, 5, 12, and 13 under 35 U.S.C. § 103(a) for obviousness over Ghori *et al.*, *Colorectal Disease* 2(2):106-112 (2000) ("Ghori") is respectfully traversed for the reasons set forth in the accompanying Ichas Declaration.

Ghori investigated telomerase inhibition using three nucleoside retroviral reverse transcriptase inhibitors ("NRTI") (*i.e.*, not NNRTIs) as a therapeutic strategy for the treatment of colorectal cancer. Ichas Declaration at ¶ 11. Despite Ghori's demonstration that the three tested NRTIs, *i.e.*, azidothymidine (AZT), dideoxythymidine (ddT), and dideoxyguanidine (ddG), inhibited telomerase activity and slowed proliferation in the tumor cell line HT29, there are a number of reasons why one of skill in the art would not have expected other NRTIs or NNRTIs to be useful for inhibiting telomerase activity and treating cell proliferation in tumor pathologies. *Id.*

Firstly, other studies investigating the potential use of NRTIs to inhibit telomerase and treat cancer have reported inconsistent results (reviewed by White et al., "Telomerase Inhibitors," *Trends in Biotech.* 19(3):114–120 (2001) ("White"). Ichas Declaration at ¶ 12. For example, Strahl and Blackburn, "Effects of Reverse Transcriptase Inhibitors on Telomere Length and Telomerase Activity in Two Immortalized Human Cells Lines," *Mol. Cell. Biol.* 16(10):53–65 (1996) ("Strahl") examined telomerase inhibition, telomere shortening, and cell growth rates in cell lines derived from B-cell lymphoma and human T-cell leukemia using the nucleoside analogs AZT, arabinofuranyl-guanosine (Ara-G), ddG, deoxyinosine (ddI), deoxyadenosine (ddA), didehydrothymidin (d4T), and the non-nucleoside RT inhibitor phosphonoformic acid (foscarnet). *Id.* Strahl reports that while ddG caused reproducible and progressive telomere shortening and telomerase inhibition, no effect on the cell population doubling rates or morphology were observed. *Id.* AZT caused progressive telomere shortening in some but not all T- and B-cell cultures, and prolonged passaging in Ara-G, ddI, ddA, d4t, or foscarnet did not

cause reproducible telomere shortening or decreased cell growth rates of viability. *Id.* Similar to the results of Strahl, Murakami et al., "Inhibition of Telomerase Activity and Cell Proliferation by a Reverse Transcriptase Inhibitor in Gynaecological Cancer Cell Lines," *Eur. J. Cancer* 35(6):1027–34 (1999) ("Murakami") reported the inhibition of telomerase activity and/or telomere length by AZT-5' triphosphate and ddI in the HEC-1 human endometrial adenocarcinoma cell line, but not the MCAS human ovarian cancer cell line. *Id.* In summarizing these divergent results, White concludes:

> [I]t is unlikely that these analogs are functioning through a selective inhibition of telomerase because their reduced proliferation is occurring in the setting of sufficiently long telomeres. A plausible explanation might be that the RTIs have a toxic effect on the cells, perhaps by inhibiting mitochondrial DNA replication leading to the observed reduction in telomerase activity, which is dose-dependent.

Id. In view of White, Strahl, and Murakami, one of skill in the art would not have had a reasonable expectation that NRTIs other than those disclosed in Ghori, and certainly not NNRTIs would be useful for inhibiting telomerase activity and slowing proliferation of tumor cells. *Id.*

Secondly, while there is at least *a priori* some rationale for testing NRTI mediated inhibition of telomerase based on their common mechanism of action, this rationale does not apply to NNRTIs which are structurally distinct and have a distinct mechanism of action. Ichas Declaration at ¶ 13. These differences are discussed more fully below. *Id.*

NRTIs are known to inhibit RT activity by the common mechanism of DNA chain termination. Ichas Declaration at ¶ 14. Indeed, any RT will incorporate the NRTI analogues into newly synthesized DNA, and once incorporated, viral DNA synthesis is halted. *Id.* Since all nucleoside analogues should bind to the catalytic domain of any RT, it might thus be expected *a priori* that a NRTI should have inhibitory activity on almost any RT. *Id.* In fact, NRTIs, such as zidovudine and lamivudine, in their 5'-triphosphate forms, exert a broad spectrum of antiviral RT activity, including, for example, inhibition of both HIV-2 and HIV-1 RTs. *Id.*

In contrast to nucleoside analogs, NNRTIs act by a completely different mode of action that involves direct binding in a reversible and non-competitive manner to a hydrophobic pocket close to the polymerase catalytic site, in the p66 subunit of RT. Ichas Declaration at ¶ 15.

Because the NNRTIs nevirapine, delavirdine, and efavirenze mediate inhibition of HIV-RT by interacting with this specific binding site on the RT enzyme, any slight variation brought about by a single point mutation can have a significant impact on inhibition. *Id.* Accordingly, other retroviral RT enzymes and some mammalian RT enzyme systems that lack this site are unaffected by these NNRTIs. *Id.*

In view of the distinct mechanisms of action of NRTIs and NNRTIs, and the specific mechanism of NNRTI mediated RT inhibition (*i.e.*, direct binding to a hydrophobic pocket in the p66 subunit of RT), one of skill in the art would have had no reasonable expectation, based on the teachings of Ghori, that NNRTIs are generally effective inhibitors of any RT, particularly the reverse transcriptase catalytic subunit of human telomerase. Ichas Declaration at ¶ 16. In fact, the general knowledge would have suggested that NNRTIs would be largely ineffective against RTs other than HIV-1 RT. *Id.* Therefore, persons of skill in the art would have lacked any expectation, based on Ghori, that NNRTIs, such as nevirapine, efavirenz, delavirdine, and compounds in the class of 5,11-dihydro-6h-dipyrido[3,2-b:2',3'-e][1,4]diazepines, would be useful for treating cell proliferation in tumor pathologies. *Id.*

Thirdly, Damm et al., "A Highly Selective Telomerase Inhibitor Limiting Human Cancer Cell Proliferation," *EMBO J.* 20(24):6958–68 (2001) ("Damm"), which identifies a class of small molecules exhibiting selective inhibition of telomerase activity without RT inhibition (*see* Figure 1C), clearly demonstrates that telomerase is distinct from other DNA and RNA polymerases, including HIV RT. Ichas Declaration at ¶ 17. Thus, a compound with known specific inhibitory activity for telomerase cannot be expected to inhibit RTs generally. *Id.*

Finally, inhibition of telomerase activity is known to induce senescence and cell death after a long lag period required for the attrition of telomeres. Ichas Declaration at ¶ 18. In contrast, the above referenced application demonstrates NNRTI-mediated differentiation of tumor cells which cannot be attributed to the inhibition of telomerase activity, because the differentiation appears too rapidly to be linked to telomerase inhibition (*see e.g.*, Example 4 and Figure 4 showing nevirapine induced differentiation of myoblasts C2C7 after 90 hours, Example 4 and Figure 5, showing nevirapine induced differentiation of teratocarcinoma F9 cells after 72 hours, and Example 5, Figure 6, showing nevirapine induced differentiation of AML cells after 5 days). *Id.* This rapid induction of cell differentiation following NNRTI exposure clearly suggests such differentiation is mediated by a mechanism other than telomerase inhibition. *Id.*

For the reasons set forth above, one of skill in the art would not conclude, based on Ghori's demonstration that certain NRTIs inhibit telomerase activity and slow cellular proliferation, that NNRTIs, a structurally distinct class of compounds with a distinct mechanism of action, would likewise exert similar effects and be useful for counteracting the loss of cellular differentiation and treating cell proliferation in tumor pathologies. Ichas Declaration at ¶ 19.

Consistent with the results in the present application and the conclusion by Dr. Ichas, three subsequent publications by one of the inventors confirm that telomerase inhibition is not implicated with the NNRTI recited in the presently claimed invention. These publications are: Sciamanna et al., "Inhibition of Endogenous Reverse Transcriptase Antagonizes Human Tumor Growth," *Oncogene* 24:3923-3931 (2005) ("Sciamanna") (attached as Exhibit B to response filed October 22, 2009); Pittoggi et al., "*In Vitro* Evidence for a New Therapeutic Approach in Renal Cell Carcinoma," *Internat'l Braz. J. Urol.* 34(4):492-502 (2008) ("Pittoggi") (copy attached as **Exhibit A**); and Landriscina et al., "Reverse Transcriptase Inhibitors Down-Regulate Cell Proliferation *in Vitro* and *in Vivo* and Restore Thyrotropin Signaling and Iodine Uptake in Human Thyroid Anaplastic Carcinoma," *J. Clin. Endocrinol. Metab.* 90(10):5663-5671 (2005) (copy attached as **Exhibit B**). Each of these publications demonstrates the rapid response induced by NNRTI. Both Sciammana (at p. 3928, right col., first full para.) and Pittoggi (at page 499, right col., first para.) clearly assert that the observed effects cannot be attributed to telomerase inhibition.

Accordingly, applicants submit that a person of ordinary skill in the art would not have considered the subject matter of claims 3, 4, 5, 12, and 13 obvious over Ghori. Therefore, this rejection should be withdrawn.

In view of the foregoing, it is submitted that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: August 19, 2010

/Edwin V. Merkel/
Edwin V. Merkel
Registration No. 40,087

NIXON PEABODY LLP
1100 Clinton Square
Rochester, New York 14604-1792
Telephone: (585) 263-1128
Facsimile: (585) 263-1600

Exhibit A: **Pittoggi et al., "*In Vitro* Evidence for a New Therapeutic Approach in Renal Cell Carcinoma," *Internat'l Braz. J. Urol.* 34(4):492-502 (2008)**

Investigative Urology

International Braz J Urol Vol. 34 (4): 492-502, July - August, 2008

In Vitro Evidence for a New Therapeutic Approach in Renal Cell Carcinoma

Carmine Pittoggi, Gianni Martis, Giorgia Mastrangeli, Bruno Mastrangeli, Corrado Spadafora

SBGSA (CP, CS), Italian National Institute of Health, Rome, Italy, Department of Urology (GM, BM), San Camillo De Lellis Hospital, Rieti, Italy and MIPI (GM), Italian National Institute of Health, Rome, Italy

ABSTRACT

Purpose: Renal cell carcinoma (RCC) is the most lethal among the common urologic malignancies, comprising 3% of all human neoplasias; approximately 40% of patients eventually die of cancer progression. One third of patients who present with metastatic disease and up to 40% treated for localized disease generally experience recurrence. RCCs are characterized by high resistance to chemo-, radio- and immunotherapy. We recently discovered an endogenous enzymatic activity, which is particularly expressed in tumorigenic cell, endogenous non-telomerase reverse transcriptase (RT) of retrotrasposon / retroviral origin, as a specific target to induce proliferation arrest in a number of human carcinogenesis in vitro culture cell lines.

Methods: To address this possibility, we have employed RCC primary cell culture testing pharmacological inhibition, in vitro, by two characterized non nucleosidic RT inhibitors, nevirapine and efavirenz; next, we assessed morphological effects and analyzed putative modulation on gene expression profile.

Results: Both treatments reduced cell proliferation rate and induced morphological differentiation and gene expression reprogramming in different RCC analyzed tumor biomarkers.

Conclusion: In this study we describe a new potential therapeutic approach to obtain considerable future benefits in renal carcinoma cure and attempt to establish a new possible pharmacological therapy based on oral drugs administration in renal RCC treatment.

Key words: *renal cell carcinoma; reverse transcriptase; gene expression; therapy*

Int Braz J Urol. 2008; 34: 492-502

INTRODUCTION

Renal cell carcinomas (RCCs) are the most frequent tumors of the kidney. They comprise 3% of all human neoplastic diseases and are increasing in incidence. The etiology of RCC remains unknown and although the majority of renal tumors develop as sporadic forms, rare familiar forms and distinct genetic abnormalities have been observed. In particular, it seems that clear cell carcinoma originates from the proximal tubular epithelium in the renal cortex. Molecular analysis of this solid tumor is furthermore complicated by the mixture of tumor cells and normal cells, composed of leukocytes and connective tissue cells. To overcome this problem the tumor cells may be adapted to grow in vitro, as primary cell cultures, in order to obtain a more homogenous cellular material to study the biochemical and molecular changes associated with neoplastic status.

RCCs can be classified into clear cell, papillary and chromophobe carcinomas based on their histological appearance.

In these tumors, a crucial role for prevention should include accurate prognosis and systematic investigation of gene expression and/or proteomic profile to identify protein changes caused by disease processes (1). The most frequent type of renal cancer is the clear cell variant, accounting for 80-85% of adult renal neoplasms.

RCCs are characterized by high resistance to chemo-, radio- and immunotherapy. Recently, new approaches, based on anti-angiogenic drugs, have emerged as potential therapies in the treatment of metastatic renal cell carcinoma (mRCC). Tyrosine kinase receptors (RTKs) are transmembrane proteins involved in signal transduction. Overexpression and/or the structural alteration of different RTKs classes are generally associated with cancer and their abnormal activation often generates cancer growth, angiogenesis and metastatization. Sunitinib malate is a molecule able to link intracellular tyrosine kinase domain of RTKs, which have an anticancer and antiangiogenetic activity. Sunitinib targets selectively vascular endothelial growth factor, KIT, Flt3 and platelet-derived growth factor receptors and the receptor encoded by the ret proto-oncogene. This drug is currently used in the treatment of gastrointestinal stromal tumors resistant to imatinib and mRCC (2). Sorafenib (Nexavar, BAY43-9006) is a new small-molecule, oral, multi-kinase inhibitor for the treatment of patients with advanced RCC. The response rate to sorafenib is partial (10%). In sorafenib treated patients there was a significant prolongation of progression-free survival. Overall survival results are still preliminary. The principal toxicities in the sorafenib patients included reversible skin rashes in 40% and hand-foot skin reaction in 30% (3). Another compound recently used in mRCC cancer therapy is temsirolimus, a specific inhibitor for the mammalian target of rapamycin kinase. Interferon alpha is widely used for mRCC but has limited efficacy and tolerability. As compared with interferon alpha, temsirolimus has been shown to improve overall survival among patients with metastatic renal-cell carcinoma and a poor prognosis (4).

This prompted us to evaluate the possibility of applying our findings for a therapeutic application in combination with kinase inhibitors recently used in mRCC, as a new class of biological agents , which have begun to break the resistance barrier (5).

Cancer progression and development have been accompanied by profound changes at cellular and molecular level, involving RNA, DNA and protein function and structure.

Recently, we have defined an endogenous activity, reverse transcriptase (RT), which is particularly expressed in tumor tissues as compared to normal tissues. In vitro cell culture treatment with specific inhibitors of the endogenous RT activity (6) has led to cell cycle arrest and induced morphological differentiation and gene expression reprogramming.

In this study, we used a human renal carcinoma derived primary cell culture to assess the cell cycle progression in vitro demonstrating that RT non nucleosidic inhibitors (nevirapine and efavirenz) induce cell cycle arrest in RCCs, morphological changes and gene markers modulation.

Our results demonstrate the treatment efficacy of these new drugs as potential therapy for in vitro RCC culture.

MATERIALS AND METHODS

Tissue Samples

Samples from 3 patients with clear RCC, containing control non tumoral ("normal") and tumor tissue were obtained from fresh nephrectomy specimens. After surgical resection the samples were immediately snap frozen in liquid nitrogen and stored at -80°C at the Department of Urology of the San Camillo De Lellis Hospital in Rieti, Italy. Paraffin sections from each specimen were reviewed by a pathologist and classified histologically according to UICC-TNM.

Primary Tissue RCC Cultures and Derived Cell Line

Autologous tumor and cortex renal tissue specimens were collected, after surgery, in cold DMEM medium containing 1% penicillin/streptomycin, 1% amphotericin, 0.5% glutamine, 20% fetal

calf serum (FCS) and kept at 4°C until processing (within 18 h). Tissues , normal and neoplastic, were vigorously washed 4-5 times vortexing in phosphate-buffered saline (PBS) pH 7.2 at 37°C and minced in 1-mm^3 fragments, in a Petri dish containing PBS, and vigorously re-washed vortexing with PBS at 37°C. The small fragments were left for 1 h in a dish in the presence of medium at 37°C and then 10 pieces were definitively plated in a new 10 cm Petri dish and covered by DMEM. Four Petri dishes were routinely prepared for each autologous tissue and incubated at 37°C in 5% CO_2. The first medium change was performed after 5 days, when the tissue fragments were removed. Cultures were fed twice weekly and passed in new dishes when 90% confluent after trypsinization and 1:2 split. Aliquots of cells were cryopreserved in 90% FCS/10% dimethyl sulfoxide and stored in liquid nitrogen after 1-2 passages. All experiments were conducted on the third passage. Primary RCC cell line obtained was well identified as T1 N0 M0 stage, II° grade, according to standard nomenclature defined by Fuhrman.

Cell Cultures

Human RCC primary culture cells were seeded in six –well plates at a density of 10^4 to 5 x 10^4 cells/well and cultured in RPMI 1640 medium with 10% fetal bovine serum. Nevirapine and efevirenz were purified from commercially available Viramune® (Boehringer-Ingelheim) and Sustiva® (Bristol-Myers Squibb) as described (7). The drugs were made 350 and 15 μM (final concentration) in dimethyl sulfoxide (DMSO, Sigma Aldrich), respectively, and added to cells 5 h after seeding, the same DMSO volume (0.2% final concentration) was added to controls. Every 48 h fresh RT inhibitors-containing medium was changed. Cells were harvested every 96 h, counted in a Burker's chamber and replated at the same density.

RNA Extraction and Semi-quantitative RT-PCR

Total RNA was extracted from RCC primary cell culture (10 x 10^6 cell) treated with nevirapine and efavirenz and untreated (CTR); the RNAeasy mini kit (Qiagen, Germany) was used. Trace amounts of contaminant chromosomal DNA was eliminated by incubation with Rnase-free Dnase I (Invitrogen, Carlsbad, CA), 1 U/μg of total RNA, for 15 min at room temperature; 200 ng of each RNA sample was used in a oligo(dT) cDNA synthesis, performed using the Thermoscript RT-PCR system (Invitrogen, USA). 2 μL for each cDNA sample were PCR amplified using the Platinum Taq DNA Polymerase I (Invitrogen, USA), in a 50 μL reaction mixture containing 30 pmoL of specific oligonucleotides (MWGBiotech, Ebersberg, Germany) in an initial 2-min step at 94°C, followed by cycles of 30 s at 94°C, 30 s at 58-62°C, 1 min at 72°C. Each oligo pair was used in sequential amplification series with increasing numbers (30-40) of cycles. PCR products were fractioned through 1.4% agarose gels and visualized with UV transilluminator light.

Set of primers used for standard PCR are designed forward (fwd) and reverse (rev). Oligonucleotide sequences and expected product sizes are listed below:

NNMT (NM 006169); PCR product size:188 bp
NNMT fwd 5'-tcaagcaggtgctgaagtgt-3'
NNMT rev 5'-atccatgatcaccaggaagc-3'
NNMT int 5'-agcacactgtgtctggatgc-3'

AFP (NM 001134); PCR product size:113 bp
AFP fwd 5'-agcttggtggtggatgaaac-3'
AFP rev 5'-tcttgcttcatcgtttgcag-3'
AFP int 5'-tccctcctgcattctctgat-3'

CD70 (EF 064709); PCR product size:110 bp
CD70 fwd 5'-aatcacacaggacctcagcaggacc-3'
CD70 rev 5'-agcagatggccagcgtcacc-3'
CD70 int 5'-agccgtagtaatggaatccca-3'

FRA2 (X 16706); PCR product size:125 bp
FRA2 fwd 5'-ccctgcacacccccatcgtg-3'
FRA2 rev 5'-tgattggtccccgctgctactgctt-3'
FRA2 int 5'-tccttagataatgcatccagtaa-3'

p 27 KIP1 (NM 004064); PCR product size: 184 bp
p 27 fwd 5'-gccctccccagtctctctta-3'
p 27 rev 5'-acagcccgaagtgaaaagaa-3'
p 27 int 5'-caggtagtttggggcaaaaa-3'

GAPDH (NM 002046); PCR product size: 650 bp
Gapdh fwd 5'-ATTCAACGGCACAGTCAAGG-3'
Gapdh rev 5'-AAGGTGGAAGAGTGGGAGTT-3'
Gapdh int 5'-GGGAAGCCCATCACCATCT-3'.

Internal oligonucleotides were used as probe to hybridize PCR DNA transferred to Hybond N+ nylon filters. After washing, filters were exposed to FUJI XR-E 30 films for the requested time.

Tumor Markers Gene Expression Profile

The induction of morphological differentiation suggests that critical regulatory genes are modulated in response to the RT inhibitory treatment. This was investigated in semi-quantitative RT-PCR analysis of cultures treated with DMSO alone, or nevirapine or efavirenz for three cycles. In RCC derived primary cell culture, we focused on a set of five genes: the CD70 gene, a new diagnostic biomarker, known as a member of the tumor necrosis factor (ligand) superfamily (8); the FRA 2 gene, Fos Related Antigen 2, associated also with apoptosis and regulator of cell proliferation, differentiation and transformation (9); the NNMT gene; the AFP gene; p27KIP1 gene.

RT-PCR results, in Figure-1, indicate that the CD70 gene and the FRA 2 gene were markedly down - regulated, in contrast with not treated tumor cells where expression levels for the same genes were considerably higher; whereas, the AFP gene, which encodes for a major plasma protein, which expression in adults is often associated with hepatoma and teratoma (10), and is thought to be the fetal counterpart of serum albumin, in RT-inhibited RCC, results in a down regulation of its mRNA expression level. In contrast, the NNMT gene, encoding for a cytokine that belongs to the tumor necrosis factor ligand family, involved in T cell antigen-presenting cell interactions, and along with CD70 shown to provide CD28-independent costimulatory signals (11), results in mRNA expression levels similar to DMSO control expression. We extended mRNA expression to another possible tumor marker: p27 KIP1. This gene encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with CDK inhibitor CDKN1A/p21. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes and thus control the cell cycle progression at G1. The degradation of this protein is required for the cellular transition from quiescence to the proliferation state. Our results show a significant p27 over-expression in nevirapine and efavirenz treated cells, confirming that the p27 expression increase was correlated to a decreasing tumor stage (12).

Thus, RT inhibitory drugs modulate the expression of critical genes implicated in the development of transformed cells, concomitantly with the induction of differentiation-like state relative to quiescence; also, this reprogramming is reversible and is abolished when RT-inhibition is released (data not shown).

Preparation of Cell-Free RCC Lysates and RT Activity Assay

Cell free RCC lysates were prepared by lysing control, nevirapine and efavirenz treated

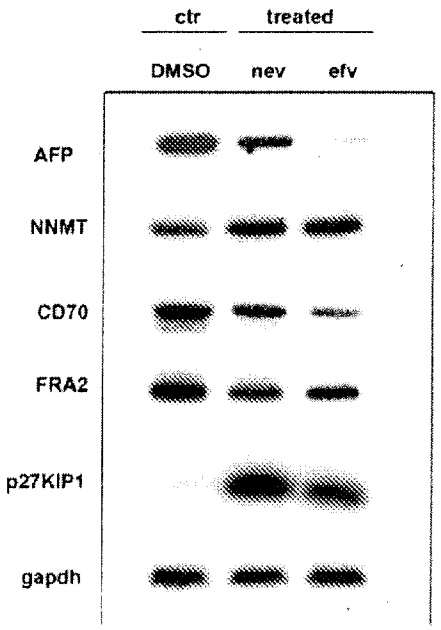

Figure 1 – RT inhibitors modulate gene expression in primary RCC cell culture. RNA extracted from cells treated with DMSO (ctr), nevirapine (nev) or efavirenz (efv), was amplified by RT-PCR, blotted and hybridized with internal oligonucleotides.

RCC cells grown till the third cycle (288 h) in vitro, using ice-cold lysis buffer (10mM Tris-Hcl, pH=7.5; 1 mM $MgCl_2$; 1mM EGTA; 0.1 mM PMSF; 5 mM β-mercaptoethanol; 0.5% CHAPS; 10% glycerol).

Cell lysates ($5x10^6$ cells /100 µL of lysis buffer) were subjected to three freeze-and-thaw (liquid nitrogen/37°C) cycles, incubated for 30 min on ice and centrifuged for 30 min at 14,000 rpm at 4°C. The supernatant containing the RT activity was aliquoted, quickly frozen in dry ice and stored at -70°C.

The protein concentration was determined by the standard Coomassie (Pierce Chemical Co., Chester, England). RT was tested in a PCR-based (PBRT) assay as previously described (3) with minor modifications. Briefly, PBRT reactions contained cells lysate aliquots corresponding to 6 ug of protein, 10 ng of bacteriophage MS2 RNA (Roche Diagnostics, Hertfordshire, England), 50 mM Tris acetate (pH 8.4), 75 mM K acetate, 40 mM Mg acetate, 5 mM DTT, 1mM of four nucleotide triphosphate mix, 2 U of Rnase OUT and 30 pmoL of MS2 reverse primer (R) (see below for the sequence) in a final volume of 20 uL. Reaction mixture were incubated at 55°C for 1 hour followed by 5 min at 85°C. One microliter of E. Coli RNaseH (2U/uL) was added to each sample and further incubated at 37°C for 20 min. Control reactions were set up by omitting cell lysate (negative controls), or adding 1 uL of ThermoScript RT (Invitrogen, Karlsruhe, Germany) 15 U/uL (positive control). Two microliters from each reaction were mixed with 30 pmoL each of forward (F) 5'-TCCTGCTCAACTTCCTGTCGAG-3' and reverse (R) 5'-CATAGGTCAAACCTCCTAGGAATG-3' MS2 primers and PCR-amplified using ThermoScript RT PCR kit (Invitrogen). PCR conditions were as follows: 95°C for 2 min; followed by 30 cycles of 94°C for 30 sec, 58°C for 45 sec and 72°C for 1 min. The amplification product is a 112-bp DNA fragment spanning positions 21-132 at the 5' end of the MS2 RNA (GeneBank J02467). PCR products were fractioned through 1.5% agarose gel electrophoresis; Southern blotted filters were hybridized with end-labeled internal oligonucleotide, 5'-TTAATGTCTTAGCGAGACGC-3'.

RESULTS

In Vitro Treatment with RT Inhibitors Induces RCCs Proliferation Arrest

The in vitro treatment with RT non-nucleosidic inhibitors induced cell growth arrest in several human cell line cultures (6) and caused, in vivo, a growth arrest in nude mice inoculated human tumor (13). In a previous work, we reported that the RT inhibitor nevirapine, largely used in anti-HIV therapy, blocks the enzymatic activity of endogenous RTs in non infected proliferating cells, as revealed using a highly sensitive RT-PCR based in vitro assay (7), and, concomitantly, reduces the growth of human primary RCC cell culture (T1 N0 M0 stage, II° grade) to prolonged exposure to RT inhibitors. Two well-defined RT inhibitors, nevirapine and efavirenz, were used. Primary culture cells were passed, counted and replated every 96 h with continuous drug re-addition (or DMSO alone in control cultures) for at least three 96 h-cycles. As shown in Figure-2, both inhibitors effectively reduced cell growth in primary cell culture, in nevirapine and efavirenz treated cells, comparing with control, with a stable effect during prolonged exposure. In Figure-2, we also show the relative rate of proliferation in nevirapine and efavirenz RCC primary culture relative to control culture (tumor derived primary RCC culture, not treated). During the first cycle, the inhibition relative to control remained at approximately 68% for nevirapine sample and 72% for efavirenz one (Figure-2). After the second and the third cycle, treated cells reduced the rate of their proliferation respectively to 31% (nevirapine treated) and 33% (efavirenz treated); it is clear that RT inhibitors were responsible for a 60% growth inhibition compared to control. We also defined the relative counts regarding cell death obtaining values similar to control (Figure-2).

RT Inhibitors Induce Differentiation in RCC Cells

It was relevant to determine whether RT inhibitors induced a differentiation-like state concomitant with reduced cell growth. We first examined RCC cells, which acquire a typical dendritic-like phenotype in response to certain inducers of differentiation (14).

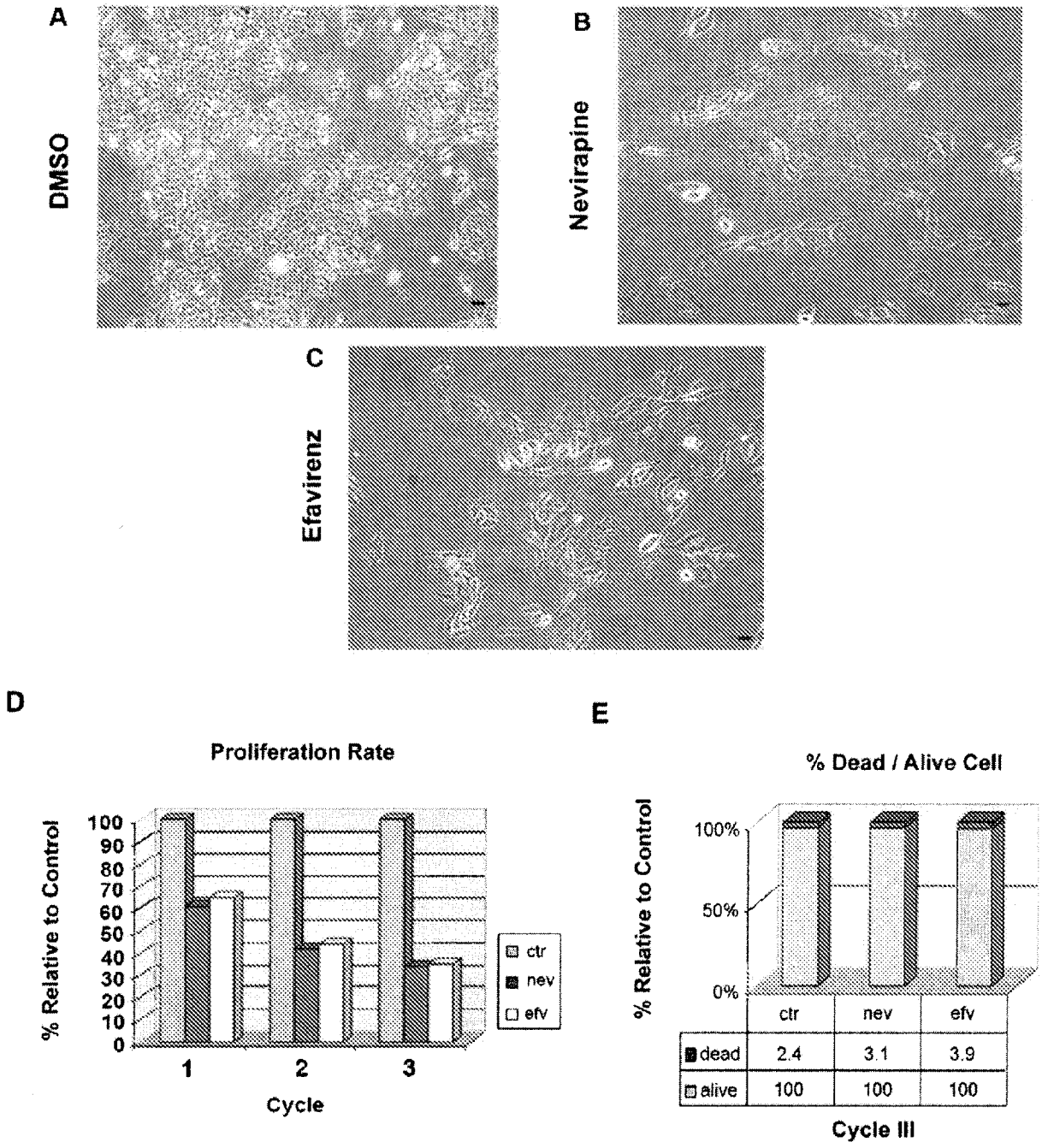

Figure 2 *– Renal carcinoma cells in vitro treatment with RT inhibitors. Treated RCC primary culture compared to ctr DMSO cultured cells (A), observed under phase-contrast microscope. Morphological differentiation in nevirapine (B) and efavirenz (C), bar, 10 µm. D) Inhibition of proliferation by anti - RT drugs: cell growth in cultures treated with DMSO (control, blue), nevirapine (nev, pink) and efavirenz (efv, white). Cells were counted and re-plated every 96 h for three cycles (1-3). Counted cells are expressed as the % of controls, taken as 100. Values represent pooled data from two experiments. E) Relative percentage of dead treated and un-treated cells relative to total alive cell; counted cells are expressed as the % total alive cell, taken as 100. Data represent medium values from three cycles.*

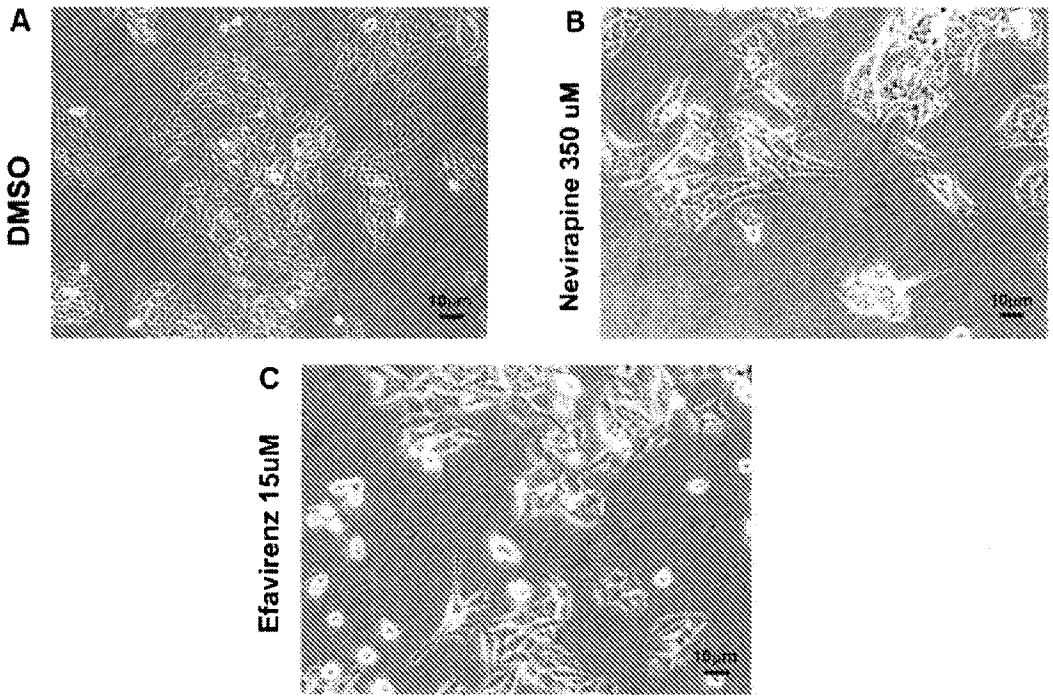

Figure 3 – Morphological differentiation of RCC in vitro primary culture after drug treatment: A) DMSO - RCC primary cell culture, control; B) In primary culture, nevirapine incubation determined dendritic like cell shape; C) As in nevirapine, also in efavirenz treated cells, there is a morphological differentiation: in this case, the dendritic extensions are less evident, but they are also present, indicating a general redistribution of cytoplasmic shape. Bars, 10 μm.

As shown in Figure-3, morphological change, revealed by cell shape and dendritic-like extensions, became evident within 4-5 days (96 h) of exposure to nevirapine or efavirenz, compared to DMSO-treated controls.

RT Enzymatic Activity Reduction in RCC Primary Cell Culture

In a previous study we detected a functional RT activity in murine F9 cell line and in several human cell line (6). To assess whether an endogenous RT is also functional in RCC cell culture, we tested the ability of cell-free lysates prepared from in vitro RCC cell culture (tissue derived primary culture), inhibitors treated and not treated (DMSO), to retrotranscribe purified MS2 phage RNA. Lysate aliquots from RCC cell line (DMSO), nevirapine treated RCC cell line and efavirenz treated RCC cell line were incubated with purified MS2 phage RNA. Incubation mixtures where then subjected to direct PCR amplification using MS2-specific oligonucleotide pairs to establish whether MS2 cDNA molecules had been newly synthesized. As shown in Figure-4, a MS2-specific cDNA product of the expected size (112 bp) was retrotranscribed from the RNA template by RCC cell line lysates (lane 1 and 2). There was no difference between the RT activity in free RCC line cell culture and RCC cell culture in presence of 0.1% DMSO.

The 112 bp-long cDNA product was only obtained when cell lysates and phage RNA were incubated together, but not when RNA (lane 7) or lysate (lane 8) were omitted from incubation mixtures.

Furthermore, we sought to establish whether RT activity was sensitive to inhibition by nevirapine and efavirenz. The mechanism of nevirapine action is well characterized and the binding site of the molecule maps a hydrophobic pocket of the RT p66 subunit,

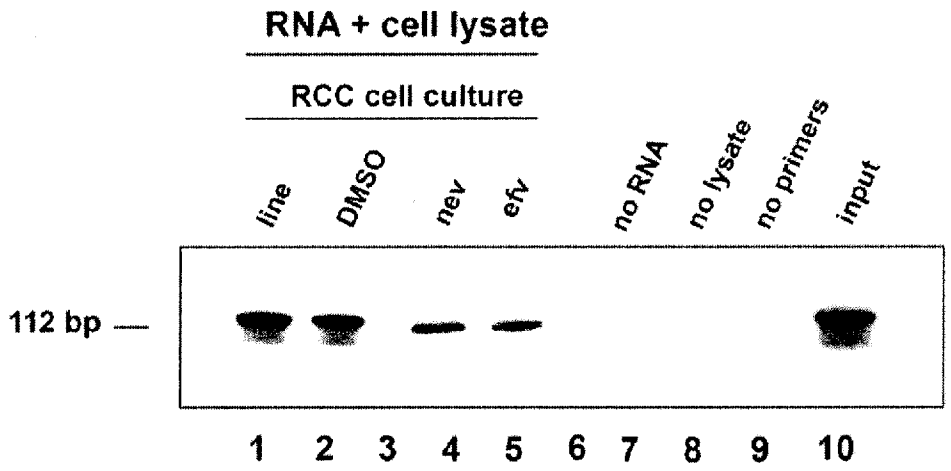

Figure 4 – Functional assay of RT activity from in vitro RCC cell culture lysates and inhibition by nevirapine and efavirenz. Endogenous RT activity was tested in vitro using MS2 phage RNA as the template and lysates from RCC primary culture cell line (lane 1) and DMSO in vitro RCC cell culture (lane 2, 4 and 5). Endogenous RT activity was tested in the absence (lane 1 and 2) or presence of RT inhibitors (nevirapine, lane 4; efavirenz, lane 5). Negative controls were selectively depleted of MS2 RNA (lane 7), cell lysate (lane 8) or PCR primers (lane 9). A positive control for the reaction utilized commercial RT instead of cell-lysate (lane 10).

near - though not overlapping with - the polymerase active site.

Preincubation of cell lysates with nevirapine and efavirenz inhibited retrotrascription of the RNA template significantly, though not totally, as shown in Figure-4, lanes 4 and 5, respectively. These results indicate that an endogenous RT activity is biologically functional in RCC in vitro cell culture and is sensitive to inhibition by nevirapine and efavirenz.

COMMENTS

This work highlights two unexpected aspects of the human genome that have implications for cancer: first, LINE L-1 elements, derived from active retroposon sequences, described by Brouha and coll. (15), have been identified as active components of a mechanism involved in the control of cell differentiation and proliferation; second, pharmacological inhibition of the endogenous RT activity which they encode, can restore control of these traits in transformed cells.

The RT inhibitors nevirapine and efavirenz, used in our study, share a common biochemical mechanism of action by binding the hydrophobic pocket in the p66 of retroviral reverse transcriptase (HIV) of RT enzymes. Although originally designed to target the HIV encoded RT, nevirapine was also able to inhibit the endogenous retro-transcriptase activity present in non infected cells (6) as shown in a highly sensitive in vitro assay (7). We also demonstrated that both drugs reduce proliferation of RCC primary cultures derived transformed cells, largely independent from cell death. Concomitant with this, RT inhibitors treatment induced a morphological transformation of the cells shape. The induction of morphological changes is rapid, different from phenotypic changes elicited by inhibitors of the telomerase-associated RT (TERT), which require an extensive treatment period which can be as long as 120 days (16).

Cytokine therapies are used in treatment of metastatic renal cell carcinoma.. However, these new biological agents only provide clinical benefit to a small subset of patients and are associated with significant toxicity (2-4). A better understanding of the

molecular biology of RCC has identified the vascular endothelial growth factor and platelet-derived growth factor signaling pathways as rational targets for anticancer therapy. The multitargeted receptor tyrosine kinase inhibitors sunitinib and sorafenib have both demonstrated improved efficacy as second-line therapy in patients with RCC. Based on these partial results, the discovery of new antitumoral targets checked by antiretroviral agents could suggest the possibility of a synergic therapy against neoplastic proliferation. Therefore, nevirapine and efavirenz could produce a cytostatic effect permitting multikinases inhibitors to enhance their tumor regression activity.

Together with growth reduction and induction of partial differentiation, RT inhibition was responsible for the reprogramming of gene expression: this implicates endogenous RT in modulation of expression for genes that promote the transition from highly proliferating, transformed phenotypes to low proliferating, differentiated phenotypes, suggesting that genome function could be the ultimate target of pharmaceutical inhibition of RT activity.

In the present study, we analyzed the expression of four genes, indicated as molecular biomarkers in RCC cells: AFP, NNMT, CD70, FRA 2 and p21KIP1 genes.

CD70 gene is strongly down-regulated in treated RCC derived primary culture, as regards control of DMSO tumor cell culture, and this pattern is very similar to differentiated renal cells; although the role of this gene in cancer development and progression remains unclear, the encoded protein (type II trans-membrane glycoprotein) seems to mediate the interaction between T and B-lymphocytes and the natural killer cell-activation; it is also implicated in processes like cell proliferation, cell to cell signaling and induction of apoptosis by binding to its receptor CD27 (17). In several studies, high expression of CD70 has been found in malignant lymphomas and nasopharyngeal tumors (18) and in all examined clear cell tumors, whereas no expression occurred in the related normal epithelial cells.

Given that RCC can be considered as an immunogenic tumor, it is tempting to speculate that a CD70 over-expression can be associated to a possible immuno-escape for clear cell RCC: a strong reduction in expression level after RT inhibitors could induce a reprogramming status where cell progression is arrested and the cell partially differentiated.

The Fos protein FRA2 forms transcription factor complexes and has been described as a regulator of cell proliferation, differentiation and transformation and in some cases the expression of FOS gene has also been associated with apoptotic cell death (19). The mRNA expression pattern of FRA2 is differently regulated in treated and untreated cell culture: in the first case, the gene was up-regulated, while in the control sample the expression level was much lower.

AFP gene encodes alpha-fetoprotein, a major plasma produced by the yolk sac and liver during fetal life. Alpha-fetoprotein expression in adults is often associated with hepatoma or teratoma. AFP mRNA is a more reliable marker of metastasis compared to serum AFP (20).

p 27 KIP1 gene encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with CDK inhibitor CDKN1A/p21. The encodec protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. The degradation of this protein, which is triggered by its CDK dependent phosphorylation and subsequent ubiquitination by SCF complexes, is required for the cellular transition from quiescence to the proliferative state.

Decreased p27 expression has been shown to be associated with aggressive tumor behavior and decreased patient survival in numerous human malignancies (12). Expression level of p27 mRNA in treated renal carcinoma cells is strongly up-regulated demonstrating that cells could reverse to a quiescent condition, prior differentiation.

Changes in gene expression are not inherited through cell division, but are reversible when RT inhibition is released (data not shown). The reversibility of examined features after release of the inhibition suggest that LINE-1 encoded RT is part of an epigenetic mechanism that can modulate gene expression and contributes to the molecular mechanisms underlying cell proliferation and differentiation.

CONCLUSIONS

The prospect of using RT inhibitors in RCC cancer therapy could have obvious advantages given

their resistance to many therapeutical approaches such as chemo-, radio- and immunotherapy.

The finding of a stable inhibition of endogenous reverse transcriptase activity in tumor or proliferative RCC cells opens the possibility for the involvement of retro-elements and retrotrasposon-sequences in the control of the proliferative process. In this study, we attempted to establish a new therapeutic approach to arrest in vitro cell growth in a RCC-derived primary cell culture as a possible useful application in cancer treatment.

ACKNOWLEDGEMENT

This work was supported by National Italian Institute of Health.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Thoenes W, Störkel S, Rumpelt HJ, Jacobi GH: Renal cell carcinoma- a classification based on cytomorphological criteria. Zentralbl Allg Pathol. 1986; 132: 503-13.
2. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, et al.: Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med. 2007; 356: 115-24.
3. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, et al.: Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med. 2007; 356: 125-34. Erratum in: N Engl J Med. 2007; 357: 203.
4. Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, Kapoor A, et al.: Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. N Engl J Med. 2007; 356: 2271-81.
5. Costa LJ, Drabkin HA: Renal cell carcinoma: new developments in molecular biology and potential for targeted therapies. Oncologist. 2007; 12: 1404-15.
6. Mangiacasale R, Pittoggi C, Sciamanna I, Careddu A, Mattei E, Lorenzini R, et al.: Exposure of normal and transformed cells to nevirapine, a reverse transcriptase inhibitor, reduces cell growth and promotes differentiation. Oncogene. 2003; 22: 2750-61.
7. Pyra H, Böni J, Schüpbach J: Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. Proc Natl Acad Sci U S A. 1994; 91: 1544-8.
8. Diegmann J, Junker K, Gerstmayer B, Bosio A, Hindermann W, Rosenhahn J, et al.: Identification of CD70 as a diagnostic biomarker for clear cell renal cell carcinoma by gene expression profiling, real-time RT-PCR and immunohistochemistry. Eur J Cancer. 2005; 41: 1794-801.
9. Shirsat NV, Shaikh SA: Overexpression of the immediate early gene fra-1 inhibits proliferation, induces apoptosis, and reduces tumourigenicity of c6 glioma cells. Exp Cell Res. 2003; 291: 91-100.
10. Giannelli G, Marinosci F, Trerotoli P, Volpe A, Quaranta M, Dentico P, et al.: SCCA antigen combined with alpha-fetoprotein as serologic markers of HCC. Int J Cancer. 2005; 117: 506-9.
11. Imura A, Hori T, Imada K, Kawamata S, Tanaka Y, Imamura S, et al.: OX40 expressed on fresh leukemic cells from adult T-cell leukemia patients mediates cell adhesion to vascular endothelial cells: implication for the possible involvement of OX40 in leukemic cell infiltration. Blood. 1997; 89: 2951-8.
12. Anastasiadis AG, Calvo-Sanchez D, Franke KH, Ebert T, Heydthausen M, Schulz WA, et al.: p27KIP1-expression in human renal cell cancers: implications for clinical outcome. Anticancer Res. 2003; 23: 217-21.
13. Sciamanna I, Landriscina M, Pittoggi C, Quirino M, Mearelli C, Beraldi R, et al.: Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. Oncogene. 2005; 24: 3923-31.
14. Sauane M, Gopalkrishnan RV, Sarkar D, Su ZZ, Lebedeva IV, Dent P, et al.: MDA-7/IL-24: novel cancer growth suppressing and apoptosis inducing cytokine. Cytokine Growth Factor Rev. 2003; 14: 35-51.
15. Brouha B, Schustak J, Badge RM, Lutz-Prigge S, Farley AH, Moran JV, et al.: Hot L1s account for the bulk of retrotransposition in the human population. Proc Natl Acad Sci U S A. 2003; 100: 5280-5.
16. Damm K, Hemmann U, Garin-Chesa P, Hauel N, Kauffmann I, Priepke H, et al.: A highly selective telomerase inhibitor limiting human cancer cell proliferation. EMBO J. 2001; 20: 6958-68.
17. Wischhusen J, Jung G, Radovanovic I, Beier C, Steinbach JP, Rimner A, et al.: Identification of CD70-mediated apoptosis of immune effector cells as a novel im-

mune escape pathway of human glioblastoma. Cancer Res. 2002; 62: 2592-9.
18. Douin-Echinard V, Bornes S, Rochaix P, Tilkin AF, Peron JM, Bonnet J, et al.: The expression of CD70 and CD80 by gene-modified tumor cells induces an antitumor response depending on the MHC status. Cancer Gene Ther. 2000; 7: 1543-56.
19. Tilghman SM: The structure and regulation of the alpha-fetoprotein and albumin genes. Oxf Surv Eukaryot Genes. 1985; 2: 160-206.
20. Yao F, Guo JM, Xu CF, Lou YL, Xiao BX, Zhou WH, et al.: Detecting AFP mRNA in peripheral blood of the patients with hepatocellular carcinoma, liver cirrhosis and hepatitis. Clin Chim Acta. 2005; 361: 119-27.

Accepted after revision:
May 23, 2008

Correspondence address:
Dr. Carmine Pittoggi
S.B.G.S.A, Italian National Institute of Health
Viale Regina Elena, 299
Rome, 00161, Italy
E-mail: carminepittoggi@hotmail.com

Exhibit B: **Landriscina et al., "Reverse Transcriptase Inhibitors Down-Regulate Cell Proliferation *in Vitro* and *in Vivo* and Restore Thyrotropin Signaling and Iodine Uptake in Human Thyroid Anaplastic Carcinoma," *J. Clin. Endocrinol. Metab.* 90(10):5663-5671 (2005)**

0021-972X/05/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 90(10):5663–5671

doi: 10.1210/jc.2005-0367

Reverse Transcriptase Inhibitors Down-Regulate Cell Proliferation *in Vitro* and *in Vivo* and Restore Thyrotropin Signaling and Iodine Uptake in Human Thyroid Anaplastic Carcinoma

Matteo Landriscina, Annarita Fabiano, Settimia Altamura, Cinzia Bagalà, Annamaria Piscazzi, Alessandra Cassano, Corrado Spadafora, Francesco Giorgino, Carlo Barone, and Mauro Cignarelli

Dipartimento di Scienze Mediche e del Lavoro, Cattedra di Oncologia Medica (M.L., S.A., A.P.), Dipartimento di Scienze Mediche e del Lavoro, Cattedra di Endocrinologia (A.F., M.C.), Centro di Medicina Molecolare (M.L., A.F., S.A., A.P., M.C.), Università degli Studi di Foggia, Foggia, Italia; Istituto di Medicina Interna, Cattedra di Oncologia Medica (C.Bag., A.C., C.Bar.), Università Cattolica del S. Cuore, Roma, Italia; Istituto Superiore di Sanità (C.S.), Roma, Italia; and Dipartimento delle Emergenze e dei Trapianti d'Organo, Cattedra di Endocrinologia (F.G.), Università di Bari, Bari, Italia

Context: Two classes of repeated genomic elements, retrotransposons and endogenous retroviruses, encode for endogenous nontelomeric reverse transcriptase (RT), a gene that is down-regulated in differentiated cells but is highly expressed in embryonic and transformed tissues. Two nonnucleosidic RT inhibitors, efavirenz and nevirapine, currently used in HIV treatment, reversibly down-regulate tumor growth and induce differentiation in several human tumor cell models.

Objectives: Aggressive biological behavior and loss of specific thyroid cell functions, such as thyroglobulin, thyroid peroxidase, TSH receptor, Na/I symporter expression, and iodine uptake are features of anaplastic thyroid cancer. Thus, we evaluated the use of RT inhibitors as a potentially differentiating and molecular-targeted treatment of this neoplasm.

Results: Our findings showed that nevirapine and efavirenz reversibly inhibit cell proliferation without triggering cell death in the undifferentiated thyroid carcinoma ARO and FRO cells, which exhibited high levels of endogenous RT activity. Inhibition of cell growth was correlated with accumulation of cells in the G_0/G_1 phase of the cell cycle, with a concomitant decrease in the S phase. Moreover, treated cells demonstrated a differentiated phenotype and a significant reprogramming of gene expression characterized by up-regulation of the TSH receptor, thyroglobulin, thyroid peroxidase, and Na/I symporter genes. Interestingly, RT inhibition reestablished the ability to uptake iodine in response to TSH either *in vitro* or *in vivo* and reversibly down-regulated tumor growth in mice xenografts of ARO cells.

Conclusions: These findings support the need for clinical trials to clarify whether RT inhibitors may restore the sensitivity to radiometabolic therapy in anaplastic thyroid tumors. (***J Clin Endocrinol Metab* 90: 5663–5671, 2005**)

RETROTRANSPOSABLE ELEMENTS, such as LINE, Alu, and endogenous retroviruses, make up at least 45% of human DNA (1). With the exception of Alu families, all other classes of retroelements are able to retrotranspose autonomously, because all are endowed with a reverse transcriptase (RT)-coding gene (2). Several studies suggest that endogenous RT activity is associated with a variety of either physiological or pathological processes. RT-coding genes are expressed at very low levels in differentiated, nonpathological tissues (3–6), whereas they are up-regulated in embryonic and undifferentiated tissues and in transformed cells (7–11). Together, these findings suggest a direct correlation between the level of expression of RT and the proliferative activity of cells (12).

We have recently observed that nevirapine and efavirenz, two nonnucleosidic RT inhibitors, widely employed in the therapy of AIDS (13), inhibit endogenous RT activity in normal and transformed mammalian cells of different histological origin (14) and induce a specific block in embryo development (15). Furthermore, either the down-regulation of the expression of RT-encoding LINE-1 elements by RNA interference or the pharmacological inhibition of RT activity results in a reversible decrease in the rate of cell growth as well as in the induction of cell differentiation in several human tumor cell lines (14, 16). RT inhibitors induce a significant reprogramming of gene expression that seems to be specific for each cell type and is responsible for the commitment of the cell to differentiate (16). Furthermore, *in vivo* efavirenz treatment significantly antagonized tumor growth in athymic xenografts of several human tumors (16).

We investigated whether pharmacological modulation of endogenous RT activity may represent a novel approach in the treatment of undifferentiated thyroid cancer, which is often metastatic and is unable to concentrate radioactive iodine (17).

First Published Online July 19, 2005

Abbreviations: DMSO, Dimethylsulfoxide; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IF, immunofluorescence; NIS, Na/I symporter; RA, retinoic acid; RT, reverse transcriptase; rTSH, recombinant TSH; TPO, thyroid peroxidase.

Materials and Methods

Cell cultures

FRO, WRO, and ARO human thyroid carcinoma cells (18) were cultured in DMEM containing 10% fetal bovine serum, glutamine, and

penicillin/streptomycin (Sigma-Aldrich, Milan, Italy). Primary cultures of normal thyroid cells were obtained from normal noninfiltrated thyroid gland collected during the surgical removal of a papillary thyroid unifocal carcinoma and processed as previously reported (19). Human recombinant TSH (rTSH) (Sigma-Aldrich) was used at a concentration of 2 mU/ml (20). Nevirapine and efavirenz were purified by Dr. Antonello Mai (University "La Sapienza," Rome, Italy) from commercially available Viramune (Boehringer-Ingelheim, Ingelheim, Germany) and Sustiva (Bristol-Myers Squibb, New York, NY) and dissolved in dimethylsulfoxide (DMSO). Both drugs, or the same volume of DMSO (0.2%, controls), were added to the cultures. Incubation was carried out continuously; RT inhibitor-containing fresh medium was changed at 48-h intervals. Evaluation of the rate of cell growth, apoptosis, necrosis, and cell cycle analysis was performed as previously reported (16).

Immunoblot analysis and RT activity assay

Cells were lysed in cold lysis buffer [20 mM Tris (pH 7.5) containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 5% (vol/vol) glycerol, 2 mM EDTA, 1% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 0.2% (wt/vol) deoxycholate]. Protein concentration was quantified using the Bio-Rad protein assay kit (Bio-Rad Laboratories GmbH, Hercules, CA) according to the manufacturer's procedures. For thyroglobulin immunoprecipitation, equal amounts of proteins were rotated for 18 h at 4 C in the presence of 1 μg mouse monoclonal antithyroglobulin antibody (Sigma-Aldrich). Protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) was added, and the samples were rotated for an additional 2 h at 4 C. Immunoprecipitated proteins were eluted by sample buffer. Equal amounts of proteins from cell lysates or immunoprecipitated proteins were resolved by 6% (wt/vol) SDS-PAGE, transferred to a nitrocellulose membrane (Hybond C; Amersham Pharmacia Biotech), and immunoblotted, respectively, with a mouse monoclonal anti-thyroid peroxidase (TPO) antibody (Alexis Biochemicals, Berne, Switzerland) or a mouse monoclonal antithyroglobulin antibody (Sigma-Aldrich). Specific bands were revealed using the Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection Kit (Amersham Pharmacia Biotech). RT activity was evaluated as previously reported (14).

Indirect immunofluorescence (IF) and confocal laser scanning microscopy. Treated and untreated cells were fixed with 4% (wt/vol) paraformaldehyde for 30 min and permeabilized in 0.1% (vol/vol) Triton X-100 and 0.1% (vol/vol) Tween 20 in PBS containing 5% (wt/vol) BSA (Sigma-Aldrich) for 1 h. F-actin staining was performed using fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma-Aldrich). IF staining was performed using mouse monoclonal antibody against human thyroglobulin (Sigma-Aldrich), TPO (21), and Na/I symporter (NIS) (22) and was revealed by FITC-conjugated IgG secondary antibody (Sigma-Aldrich). Samples were imaged under a confocal Nikon Eclipse TE 2000-S microscope (Nikon, Melville, NY). The excitation and emission wavelengths were 488 and 510 nm, respectively.

RNA extraction and semiquantitative and real-time RT-PCR analysis. Total RNA was extracted using the Trizol reagent according to the manufacturer's procedures (Invitrogen, Milan, Italy). For the first-strand synthesis of cDNA, 5 μg RNA were used in a 20-μl reaction mixture using a cDNA Superscript III (Invitrogen) according to the supplier's instructions. Oligonucleotide primers for semiquantitative and real-time PCR for TSH receptor, thyroglobulin, TPO, NIS, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table 1. Primers for real-time PCR were designed to be intron spanning. For semiquantitative PCR, 1 μl cDNA mixture was withdrawn and amplified using the Taq Gold DNA Polymerase kit (Applied Biosystems, Milan, Italy) in a Gene AMP PCR System 9700 Thermal Cycler (Applied Biosystems). Reaction conditions were 94 C for 10 min, followed by 35 cycles of 30 sec at 94 C, 30 sec at 54 C (NIS at 58 C), 5 min at 72 C, and 7 min at 72 C. β-Actin was chosen as internal control. For quantitative real-time PCR, 1 μl cDNA sample was amplified using the Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Milan, Italy) in an iCycler iQ Real Time Detection System (Bio-Rad Laboratories GmbH). Reaction conditions were 50 C for 2 min, 95 C for 2 min, followed by 50 cycles of 15 sec at 95 C, 30 sec at 56 C (TSHR at 54 C), 30 sec at 72 C. Absolute quantification was carried out against a standard curve performed by amplification of cDNA obtained from normal human thyroid. GAPDH was chosen as internal control.

TABLE 1. Sequences of primers

Genes	Primers
TSH receptor	
Semiquantitative PCR	
Sense 1411–1435	GCCTCTGTAGACCTCTACACTCAC
Antisense 2010–2028	CTTTTCAATCAGTTCATAGACATC
Real-time PCR	
Sense 93–112	CCATCAGGAGGAGGACTTCA
Antisense 212–231	ATTGGGCAGATTAGAAAATG
Thyroglobulin	
Semiquantitative PCR	
Sense 2102–2123	CAGAGTGCTACTGTGTTGATGC
Antisense 2787–2808	TCATCCACACACCAGCAGTTCC
TPO	
Semiquantitative PCR	
Sense 325–344	ACTCAACAATCACAGCATCC
Antisense 725–742	TGCTCTGTGGTGTGAACG
NIS	
Semiquantitative PCR	
Sense 676–696	GCCCTCATCCTGAACCAAGTG
Antisense 889–907	TGATCCGGGAGTGGTTCTG
Real-time PCR	
Sense 1742–1762	CCATCCTGGATGACAACTTGG
Antisense 1821–1841	AAAAACAGACGATCCTCATTG
GAPDH	
Real-time PCR	
Sense 180–197	CAAGGCTGAGAACGGGGAA
Antisense 250–269	GCATCGCCCCACTTGATTTT
β-Actin	
Semiquantitative PCR	
Sense 1023–1041	GGCATCGTGATGGACTCCG
Antisense 1824–1842	GCTGGAAGGTGGACAGCGA

In vitro iodine uptake assay. Iodine uptake assay was performed as reported by Schmutzler *et al.* (20). ARO and FRO cells were incubated for 10 d in the presence and absence of, respectively, 10 and 20 μM efavirenz and 350 μM nevirapine, then harvested, counted, plated in 24-well plates, and further incubated for 48 h in the same conditions in the presence and absence of 2 mU/ml human rTSH. For the assay, the medium was removed and washed with 1 ml HBSS (137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.55 mM glucose, 10 mM HEPES, pH 7.3). Cells were overlaid with HBSS containing 10 μM NaI and carrier-free $Na^{125}I$ to give a specific activity of 20 mCi/mmol. To control the specific uptake, some of the reactions were supplemented with the NIS inhibitor $NaClO_4$ (10 μM). After 30 min at 37 C in a humid atmosphere, cells were washed with ice-cold HBSS, and accumulated iodine was extracted at −20 C with 1 ml ethanol. Ethanol extracts were counted in a Packard Cobra II Auto-γ counter (PerkinElmer, Wellesley, MA). In parallel cell cultures, incubated in the same conditions, cells were harvested and counted in a Burker chamber (Sigma-Aldrich). Iodine uptake was normalized by cell numbers and expressed as cpm/100,000 cells. Results represent the average (±SD) of three experiments, each in quadruplicate.

Tumor xenografts, animal treatment, and in vivo iodine uptake assay. Athymic nude 4-wk-old mice (Harlan, Milan, Italy) were kept in accordance with European community guidelines. Mice were inoculated sc in the lower back with ARO cells (1×10^6/mouse) suspended in PBS and treated as previously reported (16). The analysis of iodine accumulation *in vivo* was performed as previously reported (23). Three weeks after tumor implant, animals were injected im, twice every 24 h, with 0.5 μg of human rTSH. On the day after the last injection, 10 μCi $Na^{125}I$ were delivered ip. Animals were killed 4, 24, and 48 h after $Na^{125}I$ injection; their tumor and organs were removed and weighed. Iodine uptake was measured in a Packard Cobra II Auto-γ counter (PerkinElmer), normalized by weight, and expressed as a ratio between tumor and thyroid radioactivity.

Results

Human undifferentiated thyroid tumor cells exhibit high levels of RT activity

The endogenous RT enzymatic activity was functionally tested in an RT-PCR *in vitro* assay (14) using cell lysates as a source of endogenous RT and purified MS2 phage RNA as template. Cell lysates obtained from either anaplastic ARO or poorly differentiated FRO thyroid tumor cells were able to reverse transcribe the MS2 phage RNA, obtaining a DNA band of the expected molecular weight (Fig. 1A). Interestingly, anaplastic thyroid tumor ARO cells exhibited higher levels of endogenous RT activity than undifferentiated thyroid tumor FRO cells, whereas differentiated thyroid tumor

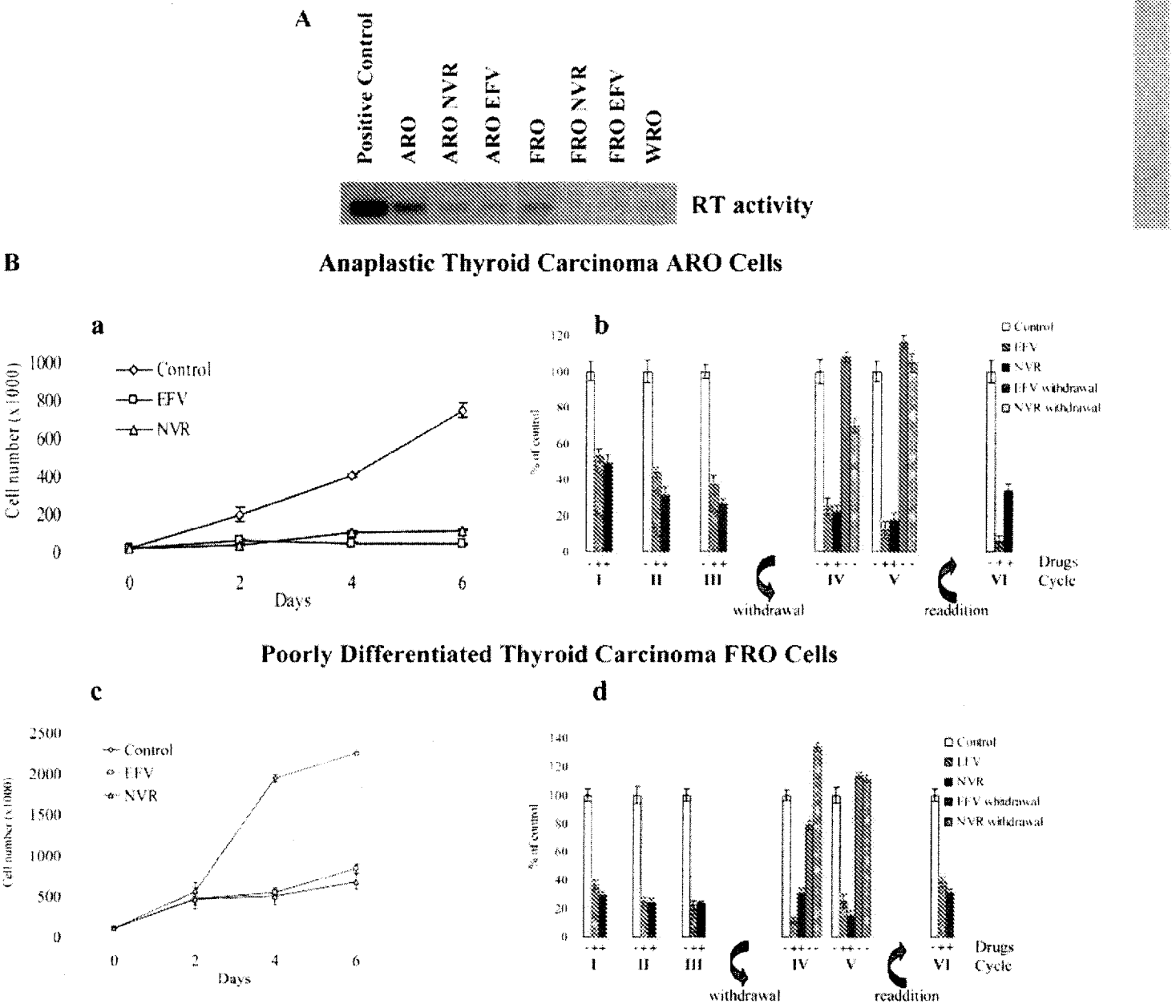

FIG. 1. A, Undifferentiated thyroid tumor ARO and FRO cells exhibit high levels of endogenous RT activity. ARO, FRO, and WRO cells were incubated in the presence of DMSO, 350 μM nevirapine (ARO NVR and FRO NVR), or 20 μM efavirenz (ARO EFV and FRO EFV). Endogenous RT activity was evaluated as reported in Ref. 14. In the positive control reaction, the cell extract was substituted with commercial RT. B, RT inhibitors reversibly down-regulate cell proliferation in undifferentiated thyroid tumor ARO and FRO cells. ARO (a) and FRO (c) cells were plated in six-well plates, incubated in the presence and absence of, respectively, 10 and 20 μm EFV or in the presence of 350 μM NVR, harvested every 2 d, and counted in a Burker chamber. Data are reported as absolute cell numbers. ARO (b) and FRO (d) cells were incubated in the presence and absence of, respectively, 10 and 20 μM EFV or in the presence of 350 μM NVR and were cultured through three subsequent cycles of 96 h each. At the end of each cycle, cells were harvested, counted in a Burker chamber, and replated at equal densities. Afterward, pretreated cells were incubated in the presence of the same concentrations of efavirenz and nevirapine or in a drug-free medium for two additional 96-h cycles. Finally, cells were resupplemented with either drug for an additional cycle. Data are reported as percentage of control cells.

WRO cells did not reveal detectable levels of RT activity. Moreover, the treatment of ARO and FRO cells with 350 μM nevirapine and 20 μM efavirenz significantly down-regulated the levels of endogenous RT (Fig. 1A). These findings suggest that undifferentiated thyroid tumor cells are characterized by high levels of RT activity and that this activity is down-regulated in the presence of pharmacological inhibitors of RT.

RT inhibitors reversibly down-regulate the rate of cell growth in human undifferentiated thyroid tumor cells without inducing apoptosis or necrosis

To reveal the rate of apoptosis and necrosis in ARO and FRO thyroid carcinoma exposed to RT inhibitors, cells were incubated in the presence and absence of 5, 10, 20, and 40 μM efavirenz or 200, 350, and 500 μM nevirapine, harvested after 72 h and stained with Hoechst 33258 and propidium iodate. We did not observe any increase in cell death in the presence of 1) 10 μM efavirenz in ARO cells, 2) 20 μM efavirenz in FRO cells, and 3) 350 μM nevirapine in both cell lines. By contrast, a 15–20% and 5–10% induction, respectively, of apoptosis and necrosis were observed with higher doses of either drug. Of note, these noncytotoxic concentrations of efavirenz (10–20 μM) and nevirapine (350 μM) are in the therapeutic range of both drugs (24). Thus, ARO and FRO thyroid tumor cells were cultured in the presence and absence of, respectively, 10 or 20 μM efavirenz or 350 μM nevirapine, harvested every 2 d, and counted. As reported in Fig. 1B, the growth rate of ARO (a) and FRO (c) cells was significantly inhibited by either drug.

Furthermore, we addressed the question of whether RT-dependent inhibition of cell growth is reversible. Both cell lines were incubated in the presence and absence of either drug and cultured for three 96-h cycles. Cells were harvested every cycle, counted, and replated at equal densities. The continuous incubation of cells with either drug produced about 60–70% inhibition of the rate of cell proliferation (Fig. 1B, b and d). Afterward, pretreated cells were incubated in the presence of either efavirenz or nevirapine or else in a drug-free medium. The withdrawal of RT inhibitors resulted in a prompt recovery of cell proliferation with a rate of cell growth similar to control cells. Moreover, the down-regulation of cell proliferation was reinduced when the same ARO and FRO cell cultures were resupplemented with RT inhibitors (Fig. 1B, b and d). These findings suggest that RT may be involved in the regulation of cell proliferation in undifferentiated thyroid cancer cells.

RT inhibitors enhance the percentage of ARO and FRO cells in the G_0-G_1 phase of cell cycle

We further investigated the cell cycle distribution in thyroid tumor cell lines treated with efavirenz and nevirapine. Both RT inhibitors induced a significant increase in the percentage of cells in the G_0-G_1 phase of the cell cycle from 39.3 ± 0.9 in control ARO cells to 62.4 ± 2.8 and 65.9 ± 2.4 in ARO cells exposed, respectively, to nevirapine and efavirenz and from 32.9 ± 2.7 in control FRO cells to 46.9 ± 3.0 and 42.8 ± 3.4, respectively, in nevirapine- and efavirenz-treated FRO cells. Moreover, we observed a parallel decrease in cells in the S phase from 50.2 ± 2.2 in control ARO cells to 23.2 ± 2.9 and 25.8 ± 1.0 in ARO cells treated, respectively, with nevirapine and efavirenz and from 39.5 ± 1.2 in control FRO cells to 24.1 ± 2.3 and 22.6 ± 1.9 in FRO cells exposed, respectively, to nevirapine and efavirenz. Interestingly, the withdrawal of efavirenz and nevirapine from the cell culture for 4 d resulted in the loss of the G_0-G_1 phase fraction accumulation. This observation suggests that down-regulation of RT activity correlates with the arrest of cells in the G_0 phase or with a delay in G_1-S transition.

RT inhibitors induce cell differentiation in human anaplastic thyroid tumor cells

Thyroid tumor ARO cells exhibit several morphological features representative of anaplastic tumors, such as high proliferation rate and formation of cell clusters organized into multilayer populations, whereas the poorly differentiated thyroid carcinoma FRO cells are devoid of most of these features (Fig. 2). We questioned whether RT inhibition is able to revert the morphological features of anaplasia in ARO cells, as previously demonstrated in human melanoma and prostate carcinoma cells treated with RT inhibitors (16). Thus, ARO and FRO cells were incubated in the presence of DMSO or 350 μM nevirapine for 4 d, harvested, replated at high density, and further cultured for 2 d in the same conditions. Nevirapine treatment induced in anaplastic thyroid

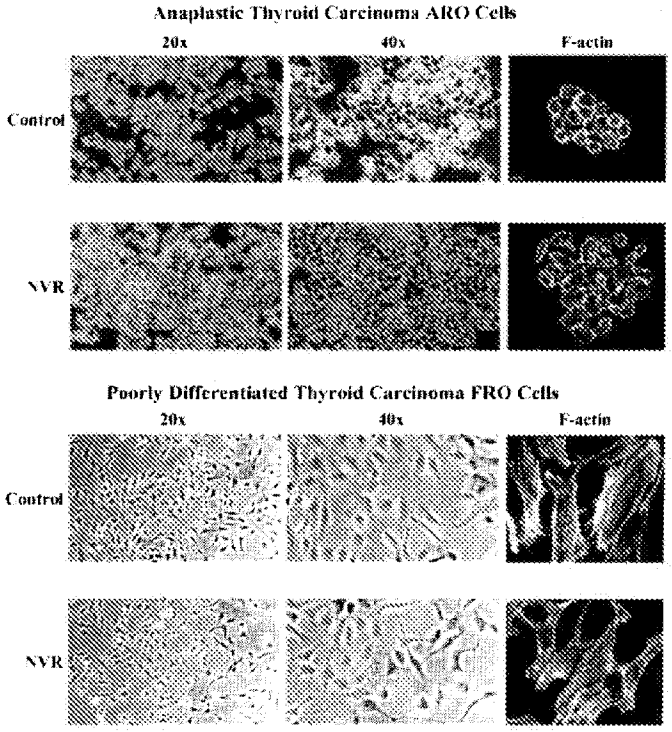

FIG. 2. Nevirapine (NVR) induces morphological differentiation in anaplastic thyroid tumor ARO cells. ARO and FRO cells were cultured in the presence of DMSO (Control) or 350 μM NVR for 4 d, harvested, counted, replated at high density in six-well plates, and further incubated in the same medium for 2 d. Photographs were obtained by phase contrast microscopy using ×20 and ×40 objectives. F-actin staining was performed using FITC-conjugated phalloidin and imaged under a confocal Nikon microscope. *Bars*, 20 μm.

tumor ARO cells 1) a more flattened phenotype, 2) an increase in cell adhesion, and 3) restoration of monolayer cell growth with a significant reduction in cluster formation (Fig. 2). By contrast, nevirapine induced only minimal morphological changes in the poorly differentiated thyroid tumor FRO cell line, with the appearance of elongated dendritic extensions tightly adherent to the substrate (Fig. 2). Similar results were obtained when the same cell lines were treated with efavirenz (data not shown). These morphological changes suggest that RT inhibitors may induce cell differentiation in anaplastic thyroid tumor cells.

We further investigated the RT-dependent differentiation of thyroid tumor cell lines by analyzing specific products of differentiated thyrocytes. Thus, ARO and FRO cells were exposed to efavirenz and nevirapine for 10 d, stained with antithyroglobulin and anti-TPO antibodies, and analyzed by indirect IF. As reported in Fig. 3A, IF analysis demonstrated undetectable expression of thyroglobulin in both ARO and FRO control cells. By contrast, exposure to efavirenz or nevirapine resulted in a significant increase in thyroglobulin levels. Consistently, ARO and FRO untreated cells exhibited undetectable levels of TPO, whereas the exposure of ARO

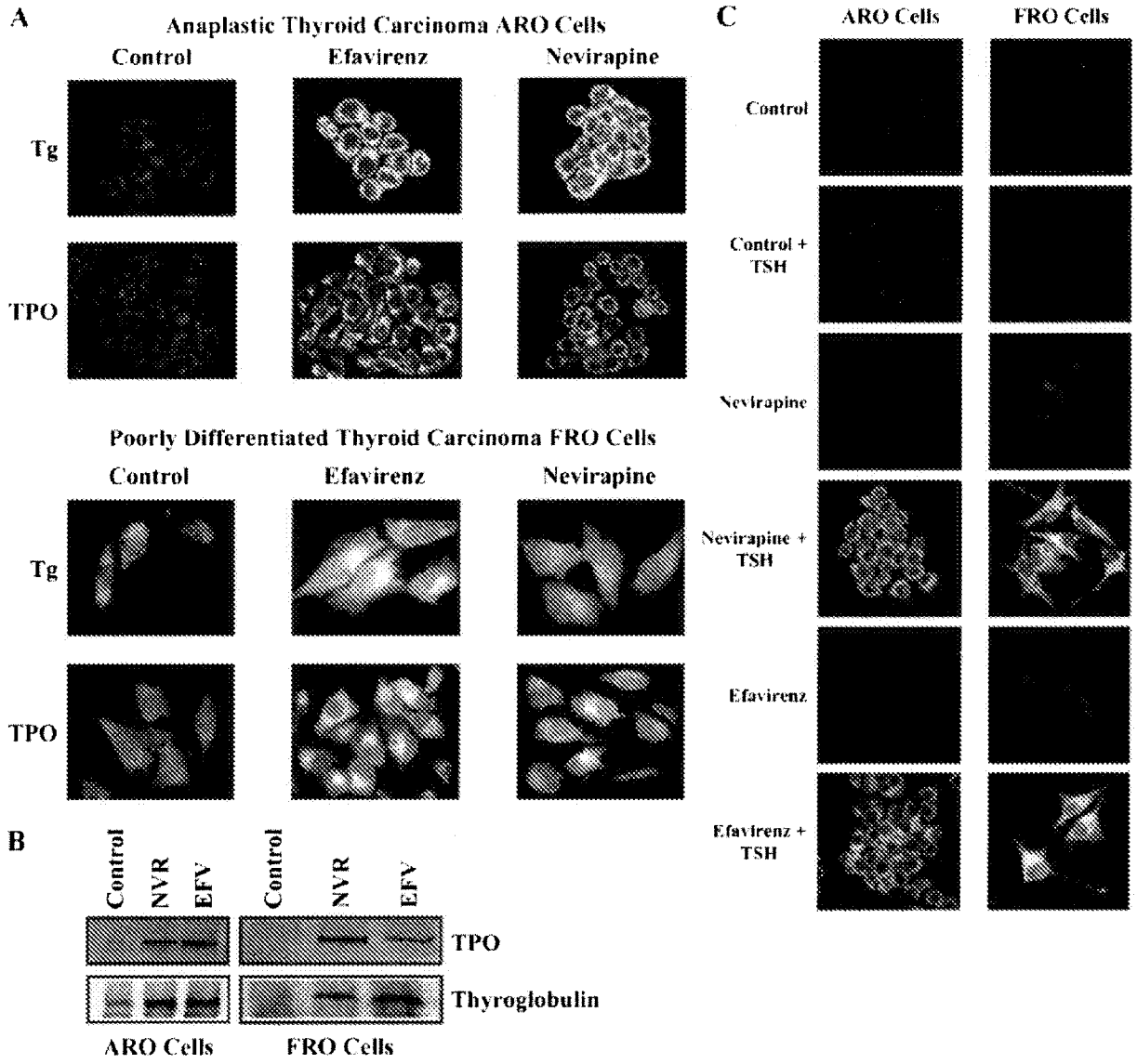

FIG. 3. RT inhibitors induce the expression of thyroglobulin, TPO, and NIS in undifferentiated thyroid tumor ARO and FRO cells. A, ARO and FRO cells were incubated in the presence of DMSO (Control), efavirenz, or nevirapine for 10 d, fixed, permeabilized, and stained by a mouse monoclonal antibody against human thyroglobulin (Tg) or human TPO. Specific signal was revealed by FITC-conjugated IgG secondary antibody and imaged under a confocal Nikon microscope. B, ARO and FRO cells were incubated in the presence of DMSO (Control), efavirenz (EFV), or nevirapine (NVR) for 10 d. Total cell lysates or immunoprecipitated proteins were separated by 6% SDS-PAGE and immunoblotted, respectively, by mouse monoclonal anti-TPO or antithyroglobulin antibodies. C, ARO and FRO cells were incubated in the presence of DMSO (Control), efavirenz, or nevirapine for 4 d, stimulated with 2 mU/ml human rTSH in the presence of either drug, fixed, permeabilized, and stained by a mouse monoclonal antibody against human NIS. Specific signal was revealed by FITC-conjugated IgG secondary antibody and imaged under a confocal Nikon microscope.

and FRO cells to efavirenz or nevirapine induced a significant increase in the expression of TPO. Furthermore, TPO immunoblot analysis exhibited the expected 110-kDa band in ARO and FRO cells treated with either efavirenz or nevirapine for 10 d, but not in DMSO-treated controls (Fig. 3B). Similarly, thyroglobulin immunoprecipitation from ARO and FRO cells revealed a strong up-regulation of the expected 330-kDa band in cells exposed to efavirenz and nevirapine for 10 d (Fig. 3B). These results suggest that RT inhibitors can facilitate the onset of cell differentiation in undifferentiated thyroid tumor cell lines, as thyroglobulin and TPO genes are highly expressed by normal thyroid cells and thyroid differentiated tumors but not by anaplastic thyroid carcinoma cells (25).

RT inhibitors induce the expression of TSH receptors and restore the ability of TSH to up-regulate NIS expression in anaplastic thyroid tumors

It is well known that anaplastic thyroid tumors are characterized by reduced or absent expression of TSH receptors as well as reduced signal transduction after receptor activation (17). Thus, we questioned whether the cell differentiation obtained by pharmacological inhibition of RT was correlated with the up-regulation of TSH receptor gene expression. This was investigated by semiquantitative RT-PCR in cultures of anaplastic thyroid carcinoma ARO cells exposed to either 350 μM nevirapine or 10 μM efavirenz for 24 and 48 h. We observed that 48-h exposure to nevirapine or efavirenz induced a significant up-regulation of TSH receptor gene expression (Fig. 4A). In parallel, we also evaluated the expression of thyroglobulin and TPO genes and observed that the expression of either gene is strongly induced in nevirapine- and efavirenz-treated ARO cells. Interestingly, the up-regulation of either gene occurred within 48 h of exposure to nevirapine or efavirenz, as observed for the TSH receptor gene (Fig. 4A).

It is also well established that normal thyroid cells and differentiated thyroid tumors are able to accumulate iodine due to the expression of NIS, a gene under the control of TSH receptor signaling (25), whereas poorly differentiated and anaplastic thyroid tumors are devoid of NIS expression (17, 25). Because RT inhibitors induce differentiation in anaplastic thyroid tumor cell lines by restoring expression of the TSH receptor, we further questioned whether efavirenz and nevirapine are able to up-regulate NIS expression in a TSH-dependent manner. Therefore, ARO cells were exposed to

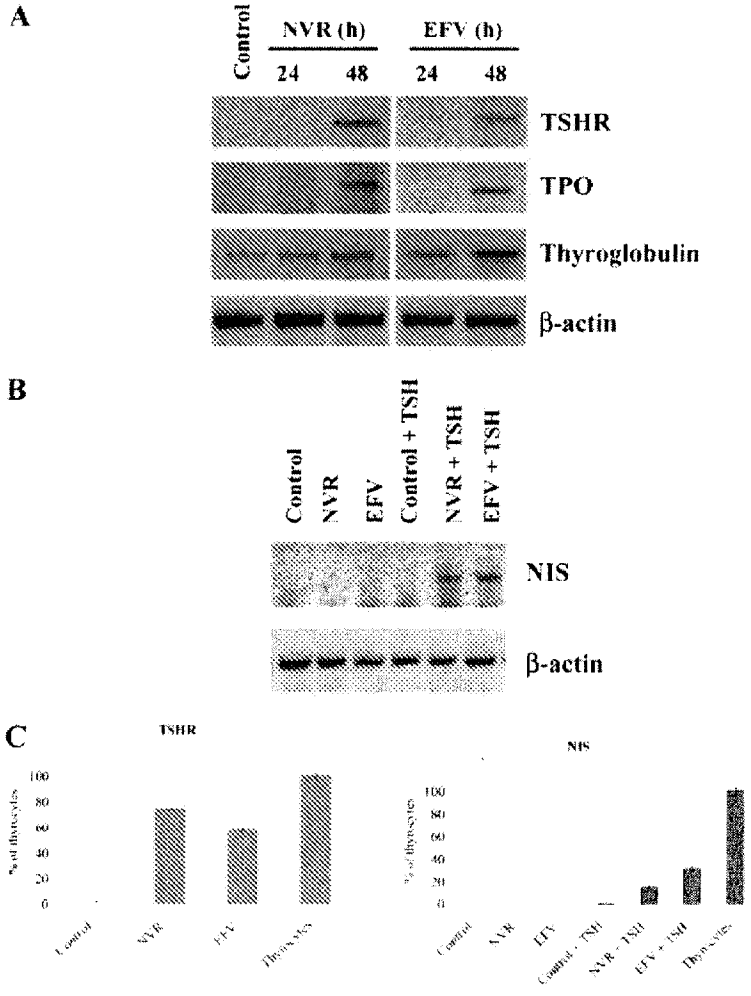

FIG. 4. RT inhibitors up-regulate TSH receptor and NIS gene expression and restore TSH signaling in anaplastic thyroid carcinoma ARO cells. A, Total RNA was extracted from ARO cells incubated for 24 and 48 h in the presence and absence of 350 μM nevirapine (NVR) or 10 μM efavirenz (EFV) and amplified by semiquantitative RT-PCR, using primers specific for TSH receptor (TSHR), thyroglobulin, and TPO genes. β-Actin was used as internal standard. B, ARO cells were incubated for 96 h in the presence and absence of 350 μM NVR or 10 μM EFV and then stimulated with 2 mU/ml human rTSH in the presence of either drug. Total RNA was amplified by semiquantitative RT-PCR using primers specific for the NIS gene. C, Total RNA was extracted either from ARO cells, exposed to 350 mM NVR or 10 μM EFV in the presence and absence of rTSH, or from normal thyrocytes and amplified by real time RT-PCR, using primers specific for the TSHR and NIS genes. GAPDH was used as internal standard. Gene expression was expressed as a percentage of that observed in normal thyrocytes.

either RT inhibitor for 4 d and then were further stimulated by TSH in the presence and absence of either drug for another 2 d. As reported in Fig. 4B, ARO cells expressed negligible levels of NIS mRNA and were not able to up-regulate the NIS gene in response to TSH. Interestingly, the basal expression of NIS was not influenced by either RT inhibitor, whereas it was significantly induced by TSH in nevirapine- and efavirenz-pretreated cells. Of note, a similar RT-dependent induction of TSH receptor, NIS, thyroglobulin, and TPO genes was also observed in FRO cells (data not shown). Consistently, IF analysis with anti-NIS antibodies in ARO and FRO cells revealed that only cells exposed to nevirapine or efavirenz, and not control cells, were able to up-regulate NIS protein levels in response to TSH (Fig. 3C). These data suggest that RT inhibitors are able to induce a specific reprogramming of gene expression in anaplastic thyroid carcinoma cells, resulting in the reestablishment of TSH signaling.

To compare the levels of expression of these thyroid-specific genes in redifferentiated ARO tumor cells with the constitutive levels of expression of the same genes in normal thyroid cells, real-time PCR was used to evaluate the expression of the TSH receptor gene in cells exposed to efavirenz, nevirapine, or DMSO for 48 h and the expression of the NIS gene in cells treated with nevirapine or efavirenz for 4 d and further stimulated with TSH for 2 d. Primary cultures of human thyrocytes were used as controls. We observed that, in comparison to control ARO cells, nevirapine and efavirenz up-regulated the TSH receptor, respectively, 7.4 and 5.8 times. Similarly, TSH was able to induce NIS expression in nevirapine- and efavirenz-pretreated cells by, respectively, 58.5 and 121.1 times in comparison with ARO control cells stimulated with TSH. Furthermore, the nevirapine- and efavirenz-induced up-regulation of the TSH receptor was, respectively, 74.1 and 58.1% of that observed in human thyrocytes, whereas the induction of NIS expression was, respectively, 15.2 and 31.5% of that observed in normal thyroid cells.

RT inhibitors restore the ability to accumulate radioactive iodine in human undifferentiated thyroid tumors

Because NIS is responsible for iodine uptake in response to TSH stimulation in thyroid cells (25), we evaluated whether ARO and FRO cells treated with RT inhibitors acquire the ability to accumulate radioactive iodine. Cells were exposed to RT inhibitors for 10 d, stimulated for 48 h with TSH to obtain the induction of the NIS gene, and further incubated in the presence of radioactive iodine. Primary cultures of normal thyroid cells were used as controls. ARO and FRO control cells exhibited minimal ability to accumulate iodine in response to TSH stimulation. Interestingly, the pretreatment of cells with efavirenz and nevirapine elicited a strong increase in iodine uptake in response to TSH (Fig. 5A). If iodine uptake was compared with the respective unstimulated controls, efavirenz treatment increased iodine uptake by about 16–17 times in ARO cells and 10 times in FRO cells, whereas nevirapine elicited a more marked increase—about 26–27 times in ARO cells and 40 times in FRO cells. The efavirenz- and nevirapine-dependent up-regulation of TSH-stimulated iodine uptake was sensitive to the NIS inhibitor, sodium perchlorate (Fig. 5A). Of note, TSH stimulation strongly up-regulated iodine uptake in normal thyroid cells, and this induction was about 3.8 times higher than that elicited by TSH in nevirapine-treated FRO cells (Fig. 5A). This ability of RT inhibitors to restore iodine uptake in anaplastic thyroid tumor cells suggests that the RT-dependent reprogramming of gene expression is able to regulate functions that are typical of differentiated cells.

RT inhibitors down-regulate tumor growth and induce iodine accumulation in mouse xenografts of ARO cell lines

Because ARO cells are well known to be highly tumorigenic in host animals (26), we evaluated the ability of efavirenz to inhibit thyroid tumor growth and induce iodine uptake *in vivo*. After inoculation of ARO cells in athymic mice, the animals were subjected to anti-RT treatment with efavirenz, and tumor size was determined every other day. Animals were treated by sc injection of efavirenz (20 mg per kilogram of body weight; 5 d/wk), starting one day after tumor inoculation (16). A 50% reduction in the growth rate was recorded in efavirenz-treated mice compared with untreated tumors (Fig. 5B). We also evaluated ARO cell-derived tumor growth in animals treated with efavirenz starting 1 d after the inoculation, but discontinuing the treatment after 14 d. In agreement with the results obtained *in vitro*, these experiments showed that inhibition of RT-dependent tumor growth is reversible *in vivo*, as it is resumed in inoculated animals in which the drug treatment was interrupted (Fig. 5B). These data indicate that RT inhibition reversibly antagonizes the growth of anaplastic thyroid tumors *in vivo*.

To evaluate iodine accumulation in ARO cells implanted in athymic mice, animals were continuously treated with DMSO or efavirenz for 3 wk, stimulated with human rTSH, and then injected with $Na^{125}I$. Iodine uptake in tumors was analyzed 4, 24, and 48 h later and was expressed as the ratio between tumor and thyroid radioactivity. As reported in Fig. 5C, efavirenz-treated tumors exhibited a progressive increase in the ratio of iodine accumulation between tumor and thyroid that was maximal 48 h after radioactive iodine injection, while control tumors exhibited a constant low level of iodine accumulation. This ability of RT inhibitors to restore iodine uptake in anaplastic thyroid tumors both *in vitro* and *in vivo* suggests that these drugs may tentatively be used to enhance the sensitivity to radiometabolic therapy in humans.

Discussion

In thyroid epithelial carcinomas, differentiation refers to the maintenance of cellular functions that are characteristic of normal thyroid follicular cells. Moreover, the degree of differentiation among thyroid tumors determines the likelihood of a beneficial response to therapeutic options that take advantage of thyroid-specific processes, such as iodine uptake and organification. Differentiated thyroid tumor cells express cell membrane receptors for TSH with active transduction machinery that stimulates progression through the cell cycle, elaboration of thyroglobulin, and both production and membrane-targeting of NIS. This provides the rationale for transient stimulation of thyroid tumors with rTSH to induce ^{131}I delivery to malignant cells for both diagnostic and

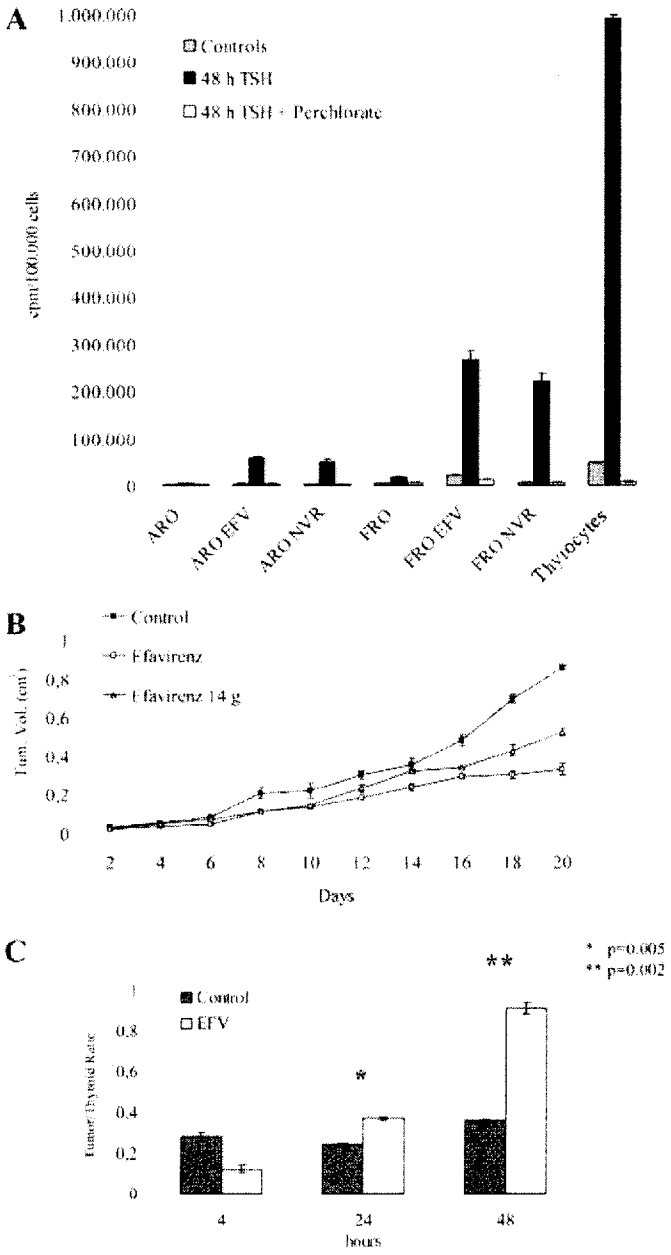

FIG. 5. RT inhibitors restore iodine uptake in undifferentiated thyroid tumor cells either *in vitro* or *in vivo* and down-regulate the growth of anaplastic thyroid tumor xenografts in athymic mice. A, ARO and FRO cells were incubated in the presence and absence of, respectively, 10 and 20 μM EFV or in the presence of 350 μM NVR for 10 d and were then harvested, counted, plated in 24-well plates, and further incubated in the same conditions in the presence and absence of 2 mU/ml human rTSH for 48 h. Primary cultures of human thyrocytes were used as positive controls and incubated in the presence and absence of 2 mU/ml human rTSH for 48 h. For the assay, the medium was removed and incubated in HBSS containing 10 μM NaI and carrier-free $Na^{125}I$. Some of the reactions received this assay buffer, supplemented with the NIS inhibitor $NaClO_4$, to control the specific uptake. Accumulated iodine was extracted with ethanol at −20 C and counted in a gamma counter. Results are normalized by cell numbers and are expressed as counts per minute per 100,000 cells. B, ARO cells were inoculated in athymic mice and injected 5 d/wk with 20 mg/kg EFV starting 1 d after tumor xenografts (*open squares*) or with DMSO (*closed squares*). In a group of animals, the treatment was discontinued 14 d after tumor injection (*open triangles*). Tumor growth was monitored by caliper measurements and reported as tumor volumes. C, Mice xenografts of ARO cells were continuously treated with 20 mg/kg EFV for 3 wk, stimulated twice with 0.5 μg of human rTSH every 24 h; on the day after the last injection, 10 μCi $Na^{125}I$ was delivered ip. Animals were killed 4, 24, and 48 h after radioactive iodine injection; iodine uptake was measured in tumors and organs, normalized by weight, and expressed as a ratio between tumor and thyroid radioactivity.

therapeutic purposes (17). The ability to treat metastatic disease with radioactive iodine is unique to thyroid cancer with sufficient expression of NIS (27). Poorly differentiated and anaplastic thyroid tumors are devoid of these cellular functions and are characterized by an aggressive biological behavior, short clinical doubling time, and invariably metastatic dissemination. The loss of thyroid-specific functions impedes both diagnostic and therapeutic efforts, due to the tumors' inability to express NIS and concentrate radioactive iodine. Dedifferentiation may result in the diminished expression of TSH receptors as well as diminished signal transduction after receptor activation. It appears likely that some of these changes that characterize the dedifferentiation of thyroid tumors, particularly epigenetic changes, may be reversible (17, 28).

The data reported in this study demonstrate that, in undifferentiated thyroid tumors, RT inhibitors produce 1) a reversible down-regulation of cell proliferation *in vitro*, 2) a reversible inhibition of tumor growth in mice xenografts, 3) an induction of cell differentiation, and 4) a reestablishment of TSH signaling and iodine uptake *in vitro* and *in vivo*. It is noteworthy that pharmacological agents are able to induce either cell differentiation and NIS expression or radioactive iodine uptake in undifferentiated thyroid tumors. Interestingly, besides normal thyroid cells and differentiated thyroid tumors, several other tissues express NIS at low levels, but this appears to be insufficient to retain radioiodine within the tumor cells long enough to deliver tumoricidal radiation doses, as shown by NIS transfection studies (29). Retention requires the organification of iodine, mediated by TPO (17). Thyroid TPO expression is diminished by malignant transformation (30) and may account for the rapid loss of accumulated radioiodine, which is probably responsible for some treatment failures (31). Retinoic acid (RA) was one of the earliest compounds tested in undifferentiated thyroid tumors with the aim of facilitating iodine uptake. Indeed, RA elicited a strong up-regulation of NIS expression in ARO cells but failed to induce iodine uptake *in vitro* and *in vivo* (20, 32). Moreover, anaplastic thyroid tumor cell lines are devoid of RA receptor β, which is likely responsible for the differentiating and antiproliferative activity of RA in differentiated thyroid tumor cells (33). Recently, histone deacetylase inhibitors have been demonstrated to induce the expression of thyroid specific genes and induce radioiodine accumulation in anaplastic thyroid tumor cells (23).

The ability of RT inhibitors to reestablish functional TSH signaling by simultaneously inducing the expression of TSH receptor, thyroglobulin, and TPO genes and the ability to respond to TSH stimulation with the up-regulation of NIS expression and iodine uptake is, to our knowledge, the first evidence that the pharmacological inhibition of RT activity

is able to induce a substantial reprogramming of cell fate in undifferentiated human tumor cells and restore functions that are typical of differentiated cells. Thus, these findings support the hypothesis that endogenous RT may represent a functional "marker" of the cellular machinery associated with high proliferation and loss of differentiation. Finally, inhibition of RT in undifferentiated thyroid tumors may be a novel molecular-targeted differentiating treatment that may be tentatively used to restore sensitivity to radiometabolic therapy. Thus, specifically designed clinical trials are needed to evaluate this hypothesis.

Acknowledgments

The authors thank Anthony Green for kindly revising the manuscript and Claudia De Tullio for expert technical support.

Received February 18, 2005. Accepted July 7, 2005.

Address all correspondence to: Dr. Matteo Landriscina, Cattedra di Oncologia Medica, Università degli Studi di Foggia, Viale Pinto, 1-71100 Foggia, Italy. E-mail: m.landriscina@unifg.it.

Address all requests for reprints to: Professor Mauro Cignarelli, Cattedra di Endocrinologia e del Metabolismo, Università degli Studi di Foggia, Viale Pinto, 1 - 71100 Foggia, Italy. E-mail: m.cignarelli@unifg.it.

This work was supported by PRIN Grant no. 2002063999_005 (to M.C.) and by Progetti di Rilevanza Nazionale Grant no. 2004054004_002 (to M.L).

References

1. **Deininger PL, Moran JV, Batzer MA, Kazazian HH** 2003 Mobile elements and mammalian genome evolution. Curr Opin Genet Dev 13:651–658
2. **Orgel LE, Crick FH** 1980 Selfish DNA: the ultimate parasite. Nature 284:604–607
3. **Kiessling AA, Goulian M** 1979 Detection of reverse transcriptase activity in human cells. Cancer Res 39:2062–2069
4. **Salganik RI, Tomsons VP, Pyrinova GB, Korokhov NP, Kiseleva EV, Khristolyubova NB** 1985 Reverse transcriptase of rat liver associated with the endogenous retrovirus related to the mouse intracisternal A-particles. Biochem Biophys Res Commun 131:492–499
5. **Medstrand P, Blomberg J** 1993 Characterization of novel reverse transcriptase encoding human endogenous retroviral sequences similar to type A and type B retroviruses: differential transcription in normal human tissues. Virology 67:6778–6787
6. **Banerjee S, Thampan RV** 2000 Reverse transcriptase activity in bovine bone marrow: purification of a 66-kDa enzyme. Biochim Biophys Acta 1480:1–5
7. **Poznanski AA, Calarco PG** 1991 The expression of intracisternal A particle genes in the preimplantation mouse embryo. Dev Biol 143:271–281
8. **Packer AI, Manova K, Bachvarova RF** 1993 A discrete LINE-1 transcript in mouse blastocysts. Dev Biol 157:281–283
9. **Mwenda JM** 1993 Biochemical characterization of a reverse transcriptase activity associated with retroviral-like particles isolated from human placental villous tissue. Cell Mol Biol 39:317–328
10. **Deragon JM, Sinnett D, Labuda D** 1990 Reverse transcriptase activity from human embryonal carcinoma cells NTera2D1. EMBO J 9:3363–3368
11. **Martin S, Branciforte D** 1993 Synchronous expression of LINE-1 RNA and protein in mouse embryonal carcinoma cells. Mol Cell Biol 13:5383–5392
12. **Ostertag EM, Kazazian HH** 2001 Biology of mammalian L1 retrotransposons. Annu Rev Genet 35:501–538
13. **Ren J, Nichols C, Bird L, Chamberlain P, Weaver K, Short S, Stuart DI, Stammers DK** 2001 Structural mechanisms of drug resistance for mutations at codons 181 and 188 in HIV-1 reverse transcriptase and the improved resilience of second generation non-nucleoside inhibitors. J Mol Biol 312:795–805
14. **Mangiacasale R, Pittoggi C, Sciamanna I, Careddu A, Mattei E, Lorenzini R, Travaglini L, Landriscina M, Barone C, Nervi C, Lavia P, Spadafora C** 2003 Exposure of normal and transformed cells to nevirapine, a reverse transcriptase inhibitor, reduces cell growth and promotes differentiation. Oncogene 22:2750–2761
15. **Pittoggi C, Sciamanna I, Mattei E, Beraldi R, Lobascio AM, Mai A, Quaglia MG, Lorenzini R, Spadafora C** 2003 Role of endogenous reverse transcriptase in murine early embryo development. Mol Reprod Dev 66:225–236
16. **Sciamanna I, Landriscina M, Pittoggi C, Quirino M, Mearelli C, Beraldi R, Mattei E, Serafino A, Cassano A, Sinibaldi-Vallebona P, Garaci E, Barone C, Spadafora C** 2005 Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. Oncogene 24:3923–3931
17. **Ain KB** 2000 Management of undifferentiated thyroid cancer. Clin Endocrinol Metab 4:615–629
18. **Lee WW, Lee B, Kim SJ, Jin J, Moon DH, Lee H** 2003 Kinetics of iodide uptake and efflux in various human thyroid cancer cells by expressing sodium iodide symporter gene via a recombinant adenovirus. Oncol Rep 10:845–849
19. **Gianoukakis AG, Cao HJ, Jennings TA, Smith TJ** 2001 Prostaglandin endoperoxide H synthase expression in human thyroid epithelial cells. Am J Cell Physiol 280:C701–C708
20. **Schmutzler C, Winzer R, Meissner-Weigl J, Kohrle J** 1997 Retinoic acid increases sodium/iodide symporter mRNA levels in human thyroid cancer cell lines and suppresses expression of functional symporter in nontransformed FRTL-5 rat thyroid cells. Biochem Biophys Res Commun 240:832–838
21. **De Micco C, Vassko V, Henry JF** 1999 The value of thyroid peroxidase immunohistochemistry for preoperative fine-needle aspiration diagnosis of the follicular variant of papillary thyroid cancer. Surgery 126:1200–1204
22. **Tonacchera M, Viacava P, Fanelli G, Agretti P, De Marco G, De Servi M, Di Cosmo C, Chiovato L, Pinchera A, Vitti P** 2004 The sodium-iodide symporter protein is always present at a low expression and confined to the cell membrane in nonfunctioning nonadenomatous nodules of toxic nodular goitre. Clin Endocrinol (Oxf) 61:40–45
23. **Furuya F, Shimura H, Suzuki H, Taki K, Ohta K, Haraguchi K, Onaya T, Endo T, Kobayashi T** 2004 Histone deacetylase inhibitors restore radioiodide uptake and retention in poorly differentiated and anaplastic thyroid cancer cells by expression of the sodium/iodide symporter thyroperoxidase and thyroglobulin. Endocrinology 145:2865–2875
24. **Kappelhoff BS, Rosing H, Huitema AD, Beijnen JH** 2003 Simple and rapid method for the simultaneous determination of the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine in human plasma using liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 792:353–362
25. **Postiglione MP, Parlato R, Rodriguez-Mallon A, Rosica A, Mithbaokar P, Maresca M, Marians RC, Davies TF, Zannini MS, De Felice M, Di Lauro R** 2002 Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland. Proc Natl Acad Sci USA 99:15462–15467
26. **Podtcheko A, Ohtsuru A, Tsuda S, Namba H, Saenko V, Nakashima M, Mitsutake N, Kanda S, Kurebayashi J, Yamashita S** 2003 The selective tyrosine kinase inhibitor, STI571, inhibits growth of anaplastic thyroid cancer cells. J Clin Endocrinol Metab 88:1889–1896
27. **Jhiang SM, Cho JY, Ryu KY, DeYoung BR, Smanik PA, McGaughy VR, Fischer AH, Mazzaferri EL** 1998 An immunohistochemical study of Na^+/I^- symporter in human thyroid tissues and salivary gland tissues. Endocrinology 139:4416–4419
28. **Nikiforov YE** 2004 Genetic alterations involved in the transition from well-differentiated to poorly differentiated and anaplastic thyroid carcinomas. Endocr Pathol 15:319–328
29. **Lee YJ, Chung JK, Shin JH, Kang JH, Jeong JM, Lee DS, Lee MC** 2004 *In vitro* and *in vivo* properties of a human anaplastic thyroid carcinoma cell line transfected with the sodium iodide symporter gene. Thyroid 14:889–895
30. **Takamatsu J, Hosoya T, Tsuji M, Yamada M, Murakami Y, Sakane S, Kuma K, Ohsawa N** 1992 Peroxidase and coupling activities of thyroid peroxidase in benign and malignant thyroid tumor tissues. Thyroid 2:193–196
31. **Filetti S, Bidart JM, Arturi F, Caillou B, Russo D, Schlumberger M** 1999 Sodium/iodide symporter: a key transport system in thyroid cancer cell metabolism. Eur J Endocrinol 141:443–457
32. **Haugen BR** 2004 Redifferentiation therapy in advanced thyroid cancer. Curr Drug Targets Immune Endocr Metabol Disord 4:175–180
33. **Elisei R, Vivaldi A, Agate L, Ciampi R, Molinaro E, Piampiani P, Romei C, Faviana P, Basolo F, Miccoli P, Capodanno A, Collecchi P, Pacini F, Pinchera A** 2005 All-*trans*-retinoic acid treatment inhibits the growth of RARβ mRNA expressing thyroid cancer cell lines but does not reinduce the expression of thyroid specific genes. J Clin Endocrinol Metab 90: 2403–2411